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STUDIES OF THE BIOCHEMICAL MECHANISM BY WHICH
6-METHYLMERCAPTOPURINE RIBONUCLEOSIDE INHIBITS TUMOR GROWTH

BY



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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "STUDIES OF THE BIOCHEMICAL MECHANISM BY WHICH 6-METHYLMERCAPTOPURINE RIBONUCLEOSIDE INHIBITS TUMOR GROWTH" submitted by Geraldine Dianne Shantz in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

6-methylmercaptopurine ribonucleoside appeared to inhibit inosinate dehydrogenase in Ehrlich ascites tumor cells in vitro, a biochemical effect of this drug which has been previously unreported. As inosinate and xanthylate do not accumulate in these cells but are readily dephosphorylated, inhibition of this enzyme caused a significant increase in the base and nucleoside formed upon dephosphorylation of inosinate derived from adenine- ^{14}C ; in addition, there was a concurrent decrease in the nucleoside formed upon dephosphorylation of xanthylate derived either from adenine- ^{14}C or hypoxanthine- ^{14}C . Inhibition of inosinate dehydrogenase by 6-methylmercaptopurine ribonucleoside was not however demonstrated in studies in vivo. A correlation was established between the amount of 6-methylmercaptopurine ribonucleoside 5'-phosphate present in Ehrlich ascites tumor cells in vivo, the magnitude of inhibition of purine biosynthesis de novo and the extent of tumor growth inhibition. These findings are consistent with the hypothesis that the biochemical mechanism of action of 6-methylmercaptopurine ribonucleoside in Ehrlich ascites tumor cells is inhibition of purine biosynthesis de novo.

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LIST OF ABBREVIATIONS

6MeMPR	6-methylmercaptapurine ribonucleoside 6-methylmercapto-9- β - <u>D</u> -ribofuranosyl-9 <u>H</u> -purine
ID ₅₀	dose of a drug which causes a 50 per cent reduction in the mass of Ehrlich ascites tumor cells at a specified dosage schedule
H.Ep.No.2	human epidermoid carcinoma number 2
FGAR	formylglycineamide ribonucleotide 5'-phosphoribosyl-formylglycineamide
PP-ribose-P	phosphoribosylpyrophosphate α - <u>D</u> -1-pyrophosphorylribofuranosyl-5-phosphate
XMP	xanthylate xanthosine 5'-monophosphate

INTRODUCTION

I INHIBITION OF TUMOR GROWTH BY 6MeMPR

A. Cell Lines Sensitive to 6MeMPR

6MeMPR inhibits the growth of H.Ep.No.2, a line of human epidermoid cancer cells grown in culture (1), and also the growth of several transplantable mouse tumors in vivo, notably the Ehrlich ascites carcinoma (2,3,4,5), Lymphoma L5178Y (6), Leukemia L1210 (1,7,8a). Adenocarcinoma 755, Adenocarcinoma R.C., and Sarcoma 180 (9).

This drug has been reported to produce a 20 to 50 per cent increase (7) and 50 to 70 per cent increase (8a) in the lifespan of mice with Leukemia L1210. When given i.p. to mice at a dose of 20 mg/Kg/day for 11 consecutive days 6MeMPR caused a 70 per cent inhibition of the growth of Adenocarcinoma 755 borne by these mice (9). The therapeutic index¹ of 6MeMPR under these conditions was less than one, indicating that the margin of safety between effective and toxic doses of this drug is small. Signs of toxicity have been reported by other investigators (2) when a dose of 20 mg/Kg of 6MeMPR was given for 5 days to mice bearing Ehrlich ascites cells.

¹ The therapeutic index in this study was the ratio between the LD10 and minimum dose for 90 per cent inhibition of tumor growth (9).

Inhibition of tumor growth by 6MeMPR in mice bearing Adenocarcinoma RC and Sarcoma 180 is less effective than in other tumors. Treatment of mice bearing these tumors with 25 mg/Kg/day of 6MeMPR for 7 consecutive days effected only 29 and 25 per cent inhibition of tumor growth respectively (9).

B. Cell Lines Sensitive to 6MeMPR and Resistant to Other Purine Analogs

6MeMPR is as effective against a line of H.Ep.No.2 cells resistant to 6-mercaptopurine as it is against the sensitive parent strain (1). When given in doses of 3 to 24 mg/Kg for 15 days, it increased the life span of mice bearing 10^4 and 10^6 Leukemia L1210 cells resistant to 6-mercaptopurine by 25 to 58 per cent and 20 to 70 per cent respectively (1).

C. Combination Chemotherapy with 6MeMPR and 6-mercaptopurine

1. Combination Chemotherapy in Transplantable Mouse Tumors

(i) Mouse Lymphoma L5178Y

Combinations of 6MeMPR and 6-mercaptopurine given simultaneously inhibit the growth of mouse Lymphoma L5178Y much more effectively than does either agent alone. The most effective combination was with 6-mercaptopurine in 5 to 10 fold molar excess (6).

(ii) Ehrlich Ascites Carcinoma

When mice bearing 4 day Ehrlich ascites tumor cells were treated simultaneously with 6MeMPR and 6-mercaptopurine (10 and 16 mg/Kg, i.p., respectively for 5 days), 9 out of 10 mice were alive and without evident tumor on the 50th day after transplantation (5). In another study, 6MeMPR was administered 6 hours prior to 6-mercaptopurine and there was a distinct increase in cytotoxicity to Ehrlich ascites carcinoma compared to a simultaneous treatment with these two drugs (4).

(iii) Leukemia L1210

Upon implantation of 10^3 L1210 cells per mouse, 50 per cent of mice treated with a combination of 6MeMPR and 6-mercaptopurine had no gross signs of leukemia 77 days after tumor implantation (8a). It was reported that this dosage administration reduced the total viable L1210 cell population to about 1 per mouse.

2. Combination Chemotherapy in Clinical Studies

6MeMPR alone was found to be inactive in patients with 6-mercaptopurine-resistant leukemia (10,6). However, when a combination of 6-mercaptopurine and 6MeMPR in a dose ratio of 3:1 (based on mg/m^2 body surface area) was given, four adults with acute myelogenous leukemia achieved complete hematologic remission (8b). This combination treatment represents an improvement over treatment with 6-mercaptopurine alone in which only 10 per cent of adults with acute myelogenous

leukemia achieve complete hematologic remission. No patient with metastatic cancer received any significant benefit from the combination. Toxic effects from this combination therapy included stomatitis, nausea, vomiting, myelosuppression, and transient abnormalities in liver function. The dose limiting toxic effect for 6MeMPR was stomatitis. It has been suggested that the unique effect of 6MeMPR on the gastrointestinal tract may indicate that combination chemotherapy might be effective against tumors of this system (8b).

II ACTIVE FORM AND METABOLISM OF 6MeMPR

A. Active Form of 6MeMPR

Many purine and pyrimidine analogs with carcinostatic activity are converted to nucleotide derivatives by tumor cells, and resistance to these drugs is sometimes accompanied by inability of the cells to form analog nucleotides. On this basis a hypothesis has been advanced that nucleotide derivatives of all such compounds are responsible for inhibition of tumor growth (1,2,11,12,13,14).

In Ehrlich ascites cells, two routes are available for the conversion of purine ribonucleosides into ribonucleotides (Chart 1). One route involves direct phosphorylation of the ribonucleoside by a purine nucleoside kinase; the other involves cleavage of the purine ribonucleoside by nucleoside phosphorylase, then reaction of the resulting purine base with PP-ribose-P catalyzed by a purine phosphoribosyltransferase. The following evidence indicates that 6MeMPR is converted to its nucleotide derivative by adenosine kinase: 6MeMPR

Nomenclature of Enzymes of Purine Metabolism

Enzyme Number (Chart 1)	Common Name	Systematic Name	E.C. Number
1	adenine phosphoribosyl- transferase	AMP:pyrophosphate phospho- ribosyltransferase	2.4.2.7
2	hypoxanthine-guanine phosphoribosyltransferase	IMP-GMP:pyrophosphate phosphoribosyltransferase	2.4.2.8
3	adenylosuccinate synthetase	IMP:L-aspartate ligase (GDP)	6.3.4.4
4	adenylosuccinate lyase	adenylosuccinate:AMP-lyase	4.3.2.2
5	AMP deaminase	AMP aminohydrolase	3.5.4.6
6	IMP dehydrogenase	IMP:NAD oxidoreductase	1.2.1.14
7	XMP aminase	XMP:L-glutamine amido-ligase (AMP)	6.3.5.2
8	GMP reductase	reduced-NADP:GMP oxido- reductase (deaminating)	1.6.6.8
9	adenylate kinase	ATP:AMP phosphotransferase	2.7.4.3
10	guanylate kinase	ATP:GMP phosphotransferase	2.7.4.8
11	nucleoside diphosphate kinase	ATP:nucleoside diphosphate phosphotransferase	2.7.4.6
12	5'-nucleotidase	5'-ribonucleotide phosphohydrolase	3.1.3.5

Nomenclature of Enzymes of Purine Metabolism (Cont'd.)

Enzyme Number (Chart 1)	Common Name	Systematic Name	E.C. Number
13	adenosine deaminase	adenosine aminohydrolase	3.5.4.4
14	adenosine kinase	ATP:adenosine 5'-phospho- transferase	2.7.1.20
15	inosine kinase	ATP:inosine 5'-phospho- transferase	-----
16	guanosine kinase	ATP:guanosine 5'-phospho- transferase	-----
17	purine nucleoside phosphorylase	purine-nucleoside:ortho- phosphate ribosyltransferase	2.4.2.1
18	xanthine oxidase	xanthine:oxygen oxidoreductase	1.2.3.2
19	guanase	guanine aminohydrolase	3.5.4.3

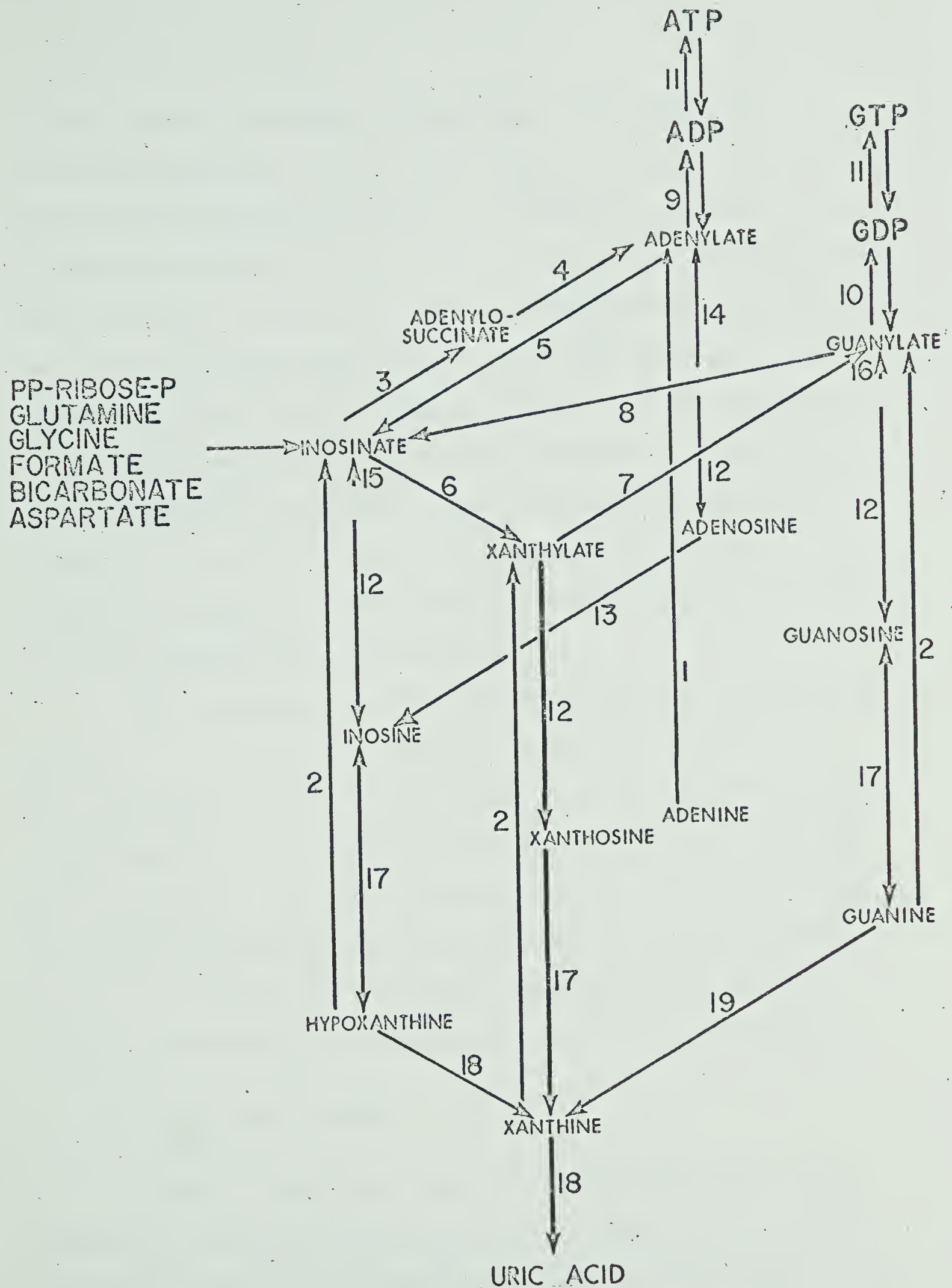


Chart 1. Outline of purine ribonucleotide metabolism.

is not converted by nucleoside phosphorylase to the free base, 6-methylmercaptapurine (1,15,16) which might participate in the phosphoribosyltransferase reaction; the optimal pH for 6MeMPR 5'-phosphate formation (5.4) is similar to the value reported for adenosine kinase, whereas the pH optima for phosphoribosyltransferases and nucleoside phosphorylases are 7.0 to 8.5 (15); substrate competition studies show that adenosine (12,15) or 2'-deoxyadenosine (15) causes marked inhibition of 6MeMPR 5'-phosphate formation, but inosine, guanosine, xanthosine, adenine or hypoxanthine (15) have no effect; cell lines of H.Ep.No.2/FA/FAR and EAC-R₂, which are resistant to 6MeMPR and to some adenosine analogs, lack adenosine kinase and cannot phosphorylate 6MeMPR (2,12).

The conversion of 6MeMPR to 6MeMPR 5'-phosphate is illustrated in Chart 2. The reaction is dependent on addition of enzyme, ATP and a divalent cation such as Mn⁺⁺ or Mg⁺⁺ (15). The addition of Co⁺⁺ or Ca⁺⁺ results in a low degree of activity, and Zn⁺⁺, Ba⁺⁺, Cu⁺⁺ and Fe⁺⁺ are inactive. It has been suggested that the absence of a proton at the N-1 of the purine ring of adenosine and 6MeMPR might be more critical for activity as a substrate for adenosine kinase than the nature of the group at the 6-position of the ring (1) (See Chart 3).

B. Metabolism of 6MeMPR

6MeMPR is relatively stable, and as mentioned above, is not degraded by nucleoside phosphorylases (1,15). 6MeMPR (15) and 6MeMPR 5'-phosphate (17) are not demethylated in tumor cells and 6MeMPR is not

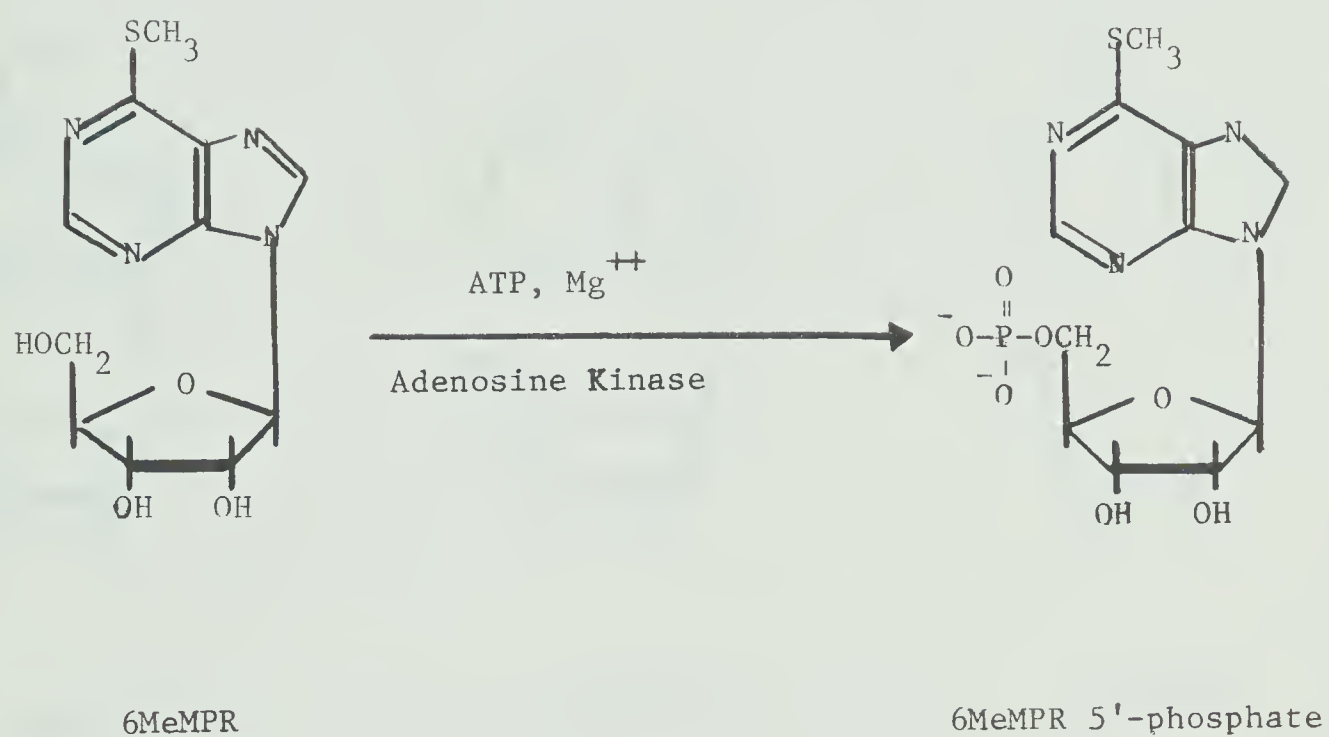
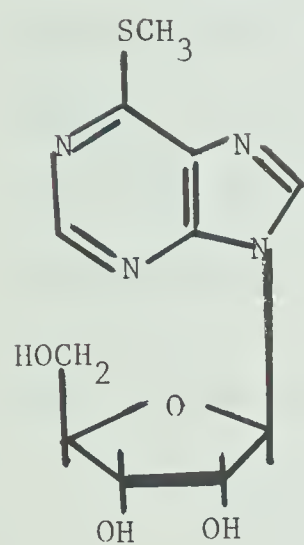
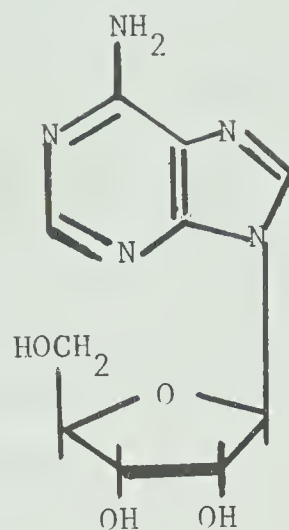


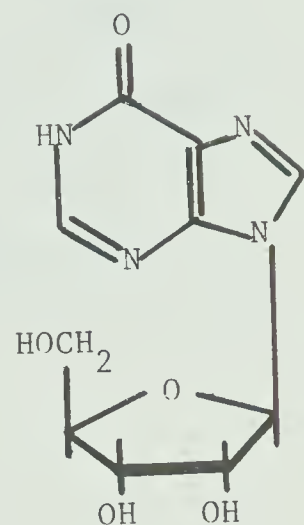
Chart 2. Enzymatic conversion of 6MeMPR to 6MeMPR 5'-phosphate.



6MeMPR



Adenosine



Inosine

Chart 3. Structural relationships between 6MeMPR, Adenosine and Inosine.

a substrate for enzymes that catabolize thiopurines, such as xanthine oxidase or aldehyde oxidase (10). 6MeMPR is not incorporated into polynucleotides (1,15). Whereas the action of adenylate kinase prevents high levels of AMP from accumulating in cells, 6MeMPR 5'-phosphate is not a substrate for this enzyme or other nucleoside monophosphate kinases, and thus it can accumulate to levels never found for AMP in vivo (13). The principal metabolite of 6MeMPR is 6MeMPR 5'-phosphate (1,15). After injection of 12 mg/Kg of 6MeMPR into mice bearing Ehrlich ascites tumors, 95 per cent of the intracellular drug was in the form of 6MeMPR 5'-phosphate (15).

III ABSORPTION AND DISTRIBUTION OF 6MeMPR

A. Absorption of 6MeMPR

1. Animal Studies

6MeMPR is readily taken up by Ehrlich ascites tumor cells. When 12 mg/Kg of radioactive 6MeMPR was injected i.p. into mice bearing Ehrlich ascites cells, more than 60 per cent of the injected radioactivity was found in the tumor cells and the radioactivity remained in the tumor for 8 hours (15). The extent of 6MeMPR uptake by several mouse tissues paralleled their adenosine kinase activity (18). There was some correlation between the uptake of 6MeMPR and the ratios of 6MeMPR 5'-phosphate to 6MeMPR, and adenosine kinase to dephosphorylating enzyme activity in BDF/1 mouse tissues (19). It was also suggested that 6MeMPR 5'-phosphate was dephosphorylated before being taken up by

tissues, and that the therapeutic effect of 6MeMPR 5'-phosphate was determined in part, by the activities of dephosphorylating enzyme and adenosine kinase (20).

2. Clinical Studies

Gastrointestinal absorption of a single i.v. injection of 6MeMPR in patients is complete (10). 6MeMPR is preferentially taken up in vivo by human red blood cells (18). Apparently uptake is by passive diffusion of 6MeMPR followed by its conversion to the more ionizable and hence less diffusible ribonucleotide, in which form the drug persists intracellularly.

B. Distribution of 6MeMPR

1. Animal Studies

After an i.p. injection of radioactive 6MeMPR into BDF/1 mice, or into mice with a solid Ehrlich tumor, the highest levels of radioactivity were found in the intestine, liver and red blood cells (15,19). Lower levels of radioactivity were found in the solid Ehrlich tumor due at least in part to limited blood circulation to this area (15). In BDF/1 mice the ratio of the drug ribonucleotide to the drug ribonucleoside remained constant for the first 48 hours in a given tissue, but varied in different tissues (19). There was very little radioactivity found in the plasma (15,19) and the drug did not cross the "blood-brain" barrier (15).

2. Clinical Studies

When patients with neoplastic disease were given a single i.v. injection of radioactive 6MeMPR (10), about 20 per cent of the dose was found in the red blood cells within 24 hours. All the drug within the red blood cells was in the form of 6MeMPR 5'-phosphate, and persisted in this form for about 6 weeks. In spite of the high concentration in the red blood cells, there was no evidence that red blood cell function and survival were adversely affected. Drug radioactivity was concentrated in the red blood cells in a 40 to 1 gradient over plasma.

Excretion of the drug was slow and sustained, about 10 per cent of the given daily dose.

IV. BIOCHEMICAL EFFECTS OF 6MeMPR

A. Inhibition of Purine Biosynthesis de novo

It is generally assumed that the site of inhibition of purine biosynthesis de novo by purine antimetabolites is the first enzyme in this pathway, phosphoribosylpyrophosphate amidotransferase (E.C.2.4.2.14) (21), although some inhibitors of this pathway also inhibit the synthesis of PP-ribose-P (22). The distinction between these two sites of action is not always made, and in some cases cannot be made in the experimental system under study. Conversion of purine bases and ribonucleosides to ribonucleotides has been shown in most, but not all cases to be a requirement for inhibition of this pathway (1,14,21,22). For example, purine base and nucleoside analogs frequently do not inhibit this pathway in cells

LIST OF ABBREVIATIONS FOR CHART 4

ABBREVIATION (Chart 4)	COMMON NAME	SYSTEMATIC NAME
PRPP	phosphoribosylpyrophosphate	α -D-1-pyrophosphorylribofuranosyl-5-phosphate
PRA	phosphoribosylamine	ribosylamine-5-phosphate
GAR	glycineamide ribonucleotide	phosphoribosyl-glycineamide
FGAR	formylglycineamide ribonucleotide	5'-phosphoribosyl-formylglycineamide
FGAM	formylglycineamidine ribonucleotide	5'-phosphoribosyl-formylglycineamidine
AIC	5-amino-4-imidazole carboxamide	
AICAR	5-amino-4-imidazole carboxamide ribonucleotide	5'-phosphoribosyl-5-amino-4-imidazole carboxamide
XMP	xanthylate	xanthosine 5'-monophosphate
AMPS	adenylosuccinate	

Nomenclature of Enzymes of Purine Metabolism

Enzyme Number (Chart 4)	Common Name	Systematic Name	E.C. Number
1	phosphoribosylpyrophosphate amidotransferase	ribosylamine-5-phosphate: pyrophosphate phosphoribosyl- transferase (glutamate amidating)	2.4.2.14
2	phosphoribosyl-formyl- glycineamide synthetase	5'-phosphoribosyl-formyl- glycineamide:L-glutamine amido- ligase (ADP)	6.3.5.3
3	IMP dehydrogenase	IMP:NAD oxidoreductase	1.2.1.14
4	hypoxanthine-guanine phosphoribosyltransferase	IMP-GMP: pyrophosphate phosphoribosyltransferase	2.4.2.8
5	adenosine kinase	ATP:adenosine 5'-phospho- transferase	2.7.1.20
6	adenine phosphoribosyl- transferase	AMP: pyrophosphate phospho- ribosyltransferase	2.4.2.7

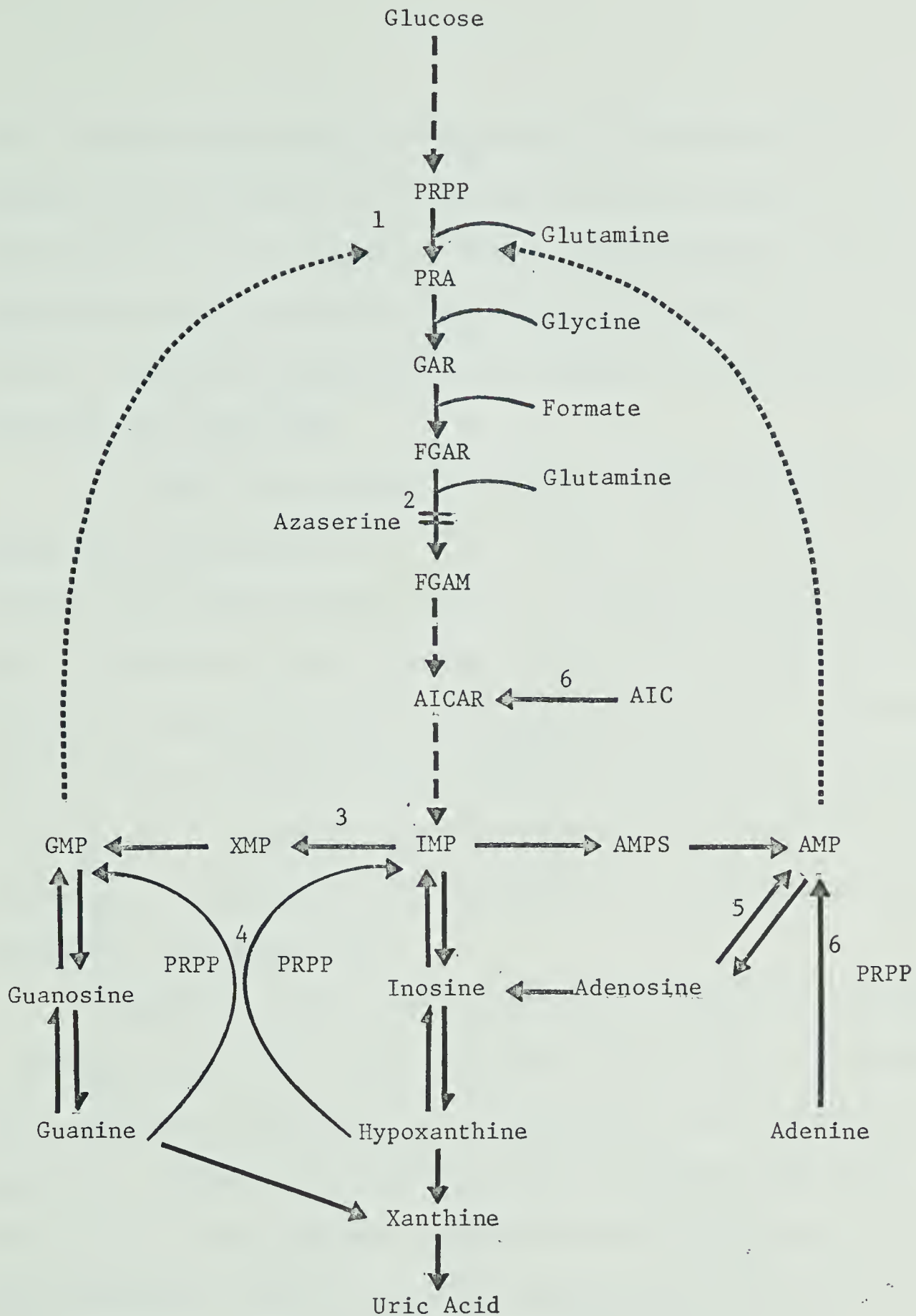


Chart 4. Purine biosynthesis de novo and purine ribonucleotide metabolism.

which lack the enzymes which convert them to ribonucleotides (2). However, a blocked nucleotide derivative of 6-mercaptopurine, bis (thioinosine) 5', 5'''-phosphate, which can circumvent the loss of hypoxanthine-guanine phosphoribosyltransferase by conversion to intracellular thioinosate by hydrolysis, was reported to inhibit purine biosynthesis de novo (22).

In pigeon liver extracts, PP-ribose-P amidotransferase was inhibited by ribonucleotides in the following order of decreasing activity: ATP > ADP > GMP > AMP > IMP > GDP. On Ehrlich ascites cells, partial inhibition of the conversion of IMP to AMP partially prevented inhibition of purine biosynthesis de novo by hypoxanthine, suggesting that IMP itself was not an active inhibitor in these cells. However, in H.Ep.No.2 cells, results were consistent with the hypothesis that IMP and guanine nucleotides were as effective as adenine nucleotides in inhibiting this pathway (23).

6MeMPR is a potent inhibitor of purine biosynthesis de novo in Ehrlich ascites tumor cells (2,3,22,24) and in lines of H.Ep.No.2 cells sensitive or resistant to 6-mercaptopurine and 6-thioguanine (1). 6MeMPR also inhibits this pathway in vivo in Leukemia L1210 cells resistant to 6-mercaptopurine. Studies with Ehrlich ascites cells in vitro showed that purine biosynthesis de novo was inhibited 75 to 83 per cent at concentrations of 6MeMPR between 10 and 100 μ M, but a concentration of adenine 500 times that of 6MeMPR was required to inhibit this pathway 36 per cent (24). In stationary or suspension cultures of H.Ep.No.2 cells sensitive or resistant to 6-mercaptopurine,

purine biosynthesis de novo was inhibited 87 to 97 per cent by 1.5 to 3 μ M 6MeMPR (1). After one i.p. injection of 25 to 98 mg/Kg of 6MeMPR into mice bearing 7 day implants of L1210 cells resistant to 6-mercaptopurine, purine biosynthesis de novo was inhibited 90 to 93 per cent (1).

In another study, an i.p. injection of 12 mg/Kg of 6MeMPR inhibited this pathway 22 to 89 per cent in normal mouse tissues (25). The degree of inhibition varied for different tissues and at different times after injection.

In intact cells, some inhibitors of purine biosynthesis de novo also inhibit the synthesis of PP-ribose-P (22). 6MeMPR causes no decrease in the rate of PP-ribose-P synthesis at concentrations of 6MeMPR which inhibit purine biosynthesis de novo by 84 per cent (22). 6MeMPR does however, inhibit phosphoribosylpyrophosphate amidotransferase, as measured by the reduced rate of utilization of PP-ribose-P in the presence of glutamine. The drug-induced accumulation of PP-ribose-P is believed to be the rationale for combination chemotherapy of 6MeMPR and 6-mercaptopurine. Prior treatment of mice bearing Ehrlich ascites tumor cells with 6MeMPR enhanced several-fold their ability to convert 6-mercaptopurine to 6-mercaptopurine ribonucleotide and their content of PP-ribose-P. These related effects were demonstrable 96 hours after a single 6MeMPR treatment. Apparently the increased nucleotide synthesis from 6-mercaptopurine is a result of "diversion" of PP-ribose-P into the phosphoribosyltransferase reaction by which 6-mercaptopurine is converted into its nucleotide derivative.

6MeMPR is a more potent inhibitor of purine biosynthesis de novo than 6-mercaptopurine (3,24,26). 6-mercaptopurine is also converted to 6MeMPR 5'-phosphate, as well as to other metabolites (17). The importance of the formation of 6MeMPR 5'-phosphate to inhibition of purine biosynthesis de novo and to the antitumor activity of 6-mercaptopurine is being presently investigated (17).

It is generally believed that PP-ribose-P amidotransferase has 2 binding sites for nucleotide inhibition, one for nucleotides of 6-aminopurines, and one for nucleotides of 6-oxypurines (17,22,27). It has been suggested that 6-mercaptopurine ribonucleotide (as an analog of IMP) and 6MeMPR 5'-phosphate (as an analog of AMP) (1) may inhibit purine biosynthesis de novo by binding to the amidotransferase at different sites (17). Evidence has however also been presented that 6MeMPR may be regarded as a substituted 6-oxypurine in this system (22).

B. Effect of 6MeMPR on Glucose Metabolism and on ADP and ATP

6MeMPR is said to be an activator of phosphofructokinase and an inhibitor of fructose-1,6-diphosphatase (13). However, even 1 mM 6MeMPR inhibits neither PP-ribose-P (22) nor lactate synthesis from glucose in Ehrlich ascites tumor cells in vitro (Dr. J. F. Henderson, personal communication). However in a study in which dosage was not reported, the drug reduced intracellular concentrations of ADP and ATP. (J. M. Oliver, and Dr. A. R. P. Paterson, unpublished results, quoted in ref. (4)) presumably due to its inhibition of purine biosynthesis de novo.

C. Effect of 6MeMPR on the Metabolism of Purine Bases

It was only recently reported that 6MeMPR inhibited the conversion of radioactive adenine, hypoxanthine and guanine into total intracellular radioactivity in Adenocarcinoma 755 cells (28). As a consequence, total nucleoside di- and tri-phosphates were reduced, as were nucleic acids formed from all three precursors. The drug was not an inhibitor of partially purified hypoxanthine-guanine phosphoribosyl-transferase from extracts of Adenocarcinoma 755 cells.

D. Effect of 6MeMPR on Purine Ribonucleoside Metabolism

6MeMPR inhibited the synthesis of inosine, uridine and guanosine by intact tumor cells (29,30). Also inhibited were inosine, uridine, and guanosine cleavage, as well as the exchange of isotope between these ribonucleosides and the corresponding C¹⁴-labeled purine bases. In Ehrlich ascites cells, 6MeMPR inhibited the metabolism of guanosine more than the metabolism of adenosine (30). The fact that the metabolism of ribonucleosides was inhibited in intact, but not in broken cell preparations suggested that 6MeMPR interfered in some way with the transport of ribonucleosides across the cell membrane.

E. Effect of 6MeMPR on the Immune Response

6MeMPR increased the survival of skin homografts in mice and inhibited the production of humoral antibodies (31). The formation of humoral antibodies was inhibited 20 and more than 90 per cent in AKR/j

mice after injection of 25 and 50 mg/Kg of 6MeMPR respectively for 3 days. 10 days after injection of 6MeMPR the level of humoral antibodies returned to normal.

V. REVERSAL OF THE GROWTH INHIBITORY EFFECTS OF 6MeMPR

If inhibition of purine biosynthesis de novo causes inhibition of tumor growth, then compounds such as aminoimidazole carboxamide or hypoxanthine that enter this pathway past the site of inhibition should be capable of reversing this growth inhibition. Aminoimidazole carboxamide or hypoxanthine protected Adenocarcinoma 755 cells growing in suspension culture against a concentration of 6MeMPR (3.5 μ M) which destroyed 75 per cent of the cells initially present (32). These findings indicate that inhibition of purine biosynthesis de novo did produce cytotoxicity. However, the failure of these compounds to protect the cells from a higher concentration of 6MeMPR suggests that this drug may have an additional site of inhibition at high doses.

VI. THESIS PROBLEM

The purpose of this research was to study the biochemical mechanism by which 6MeMPR inhibits the growth of Ehrlich ascites tumor cells. The research consisted of two parts. The first was to identify the biochemical effects of 6MeMPR. Although 6MeMPR was known to inhibit purine biosynthesis de novo, at the time this research was started there were no published results on the effect of 6MeMPR on the metabolism of purine bases. Since 6MeMPR 5'-phosphate might be considered a structural analog of AMP, IMP, GMP, or XMP, the possibility that it might inhibit one or more the enzymes of purine ribonucleotide metabolism had to be considered and this was studied.

The second part of this research was to evaluate the pharmacological significance of the biochemical effects of 6MeMPR found in the first part of the study, as well as its effect on purine biosynthesis de novo.

In order to establish which biochemical effect of a drug is its site of action, one must distinguish between those biochemical effects of the drug which cause growth inhibition, and secondary effects caused by growth inhibition. The most crucial point in evaluating the evidence is to establish a correlation between the dose of drug, the magnitude of the biochemical effect, and the extent of growth inhibition. It is also important to relate the dose of the drug to the duration of the biochemical effect and growth inhibition.

A correlation relating dose and magnitude of biochemical effects may be deduced on the basis of biochemical studies done in vitro, but must also be shown to exist on the basis of biochemical studies done in vivo and to be related to the extent of tumor growth inhibition.

VII JOURNAL STYLE

This thesis is written according to the style requested for Cancer Research. Therefore abbreviations accepted by this journal are not defined.

CHAPTER II

EXPERIMENTAL PROCEDURES

I CHEMICALS AND MATERIALS

A. Chemicals

The following were obtained from Schwarz BioResearch Inc.: adenine-8-¹⁴C (50 and 52.6 mCi/mmole); hypoxanthine-8-¹⁴C (48.4, 50, and 54.3 mCi/mmole); guanine-8-¹⁴C (31.7 mCi/mmole); and glycine-1,2-¹⁴C (50.3 and 104 mCi/mmole). ³H-labeled 6MeMPR (2564 mCi/mmole) was obtained from New England Nuclear Corp.

The Cancer Chemotherapy National Service Center provided asaserine and 6MeMPR. The following were obtained from Sigma Chemical Company: glycine, adenine, adenosine, hypoxanthine, inosine, guanine, guanosine, xanthine, xanthosine, 6MeMPR and Tris (hydroxymethyl) aminomethane. Uric acid was purchased from Calbiochem. The following were obtained from P-L Biochemicals, Inc.: AMP, ADP, ATP, GMP, GDP, GTP, IMP, and XMP.

Fisher Scientific Company supplied D-glucose, heparin sodium (167,000 units/g), potassium hydroxide and inorganic salts, in addition to materials used for chromatographic solvents. Allied Chemical supplied perchloric acid. "Fisher's medium for leukemic cells of mice" was purchased from Grand Island Biological Company as a dry powder.

B. Materials

For thin-layer chromatography, polyethyleneimine (PEI)-cellulose, on Mylar sheets, and cellulose on Mylar sheets were purchased from Canadian Laboratory Supplies and Fisher Scientific Company respectively. For paper chromatography, Whatman No. 1 paper was purchased from Fisher Scientific Company. For column chromatography, Dowex-1-formate, X8 200 to 400 mesh, was obtained from the Dow Chemical Company of Canada Ltd.

II. METHODS FOR STUDIES in vitro

A. Collection of Cells

Ehrlich ascites cells were maintained by weekly transplantation of 1 to 2×10^6 cells into female ICR Swiss mice. They were removed 6 to 7 days after transplantation and collected in Racker's buffered saline containing $25 \mu\text{g/ml}$ of heparin and 1 mg/ml of glucose in a 12 ml centrifuge tube. The composition of Racker's buffered saline is: 0.14 M NaCl , $0.01 \text{ M Tris buffer}$, $\text{pH } 7.4$, and $0.004 \text{ M Na}_2\text{HPO}_4$ buffer, $\text{pH } 7.4$. The collection of cells and subsequent manipulations were carried out at room temperature. The cells were washed 3 times with the above solution, and after a 7 minute centrifugation at $2,000 \text{ rpm}$, were gently suspended in 18 volumes of Fisher's medium containing sodium bicarbonate and glucose to give the concentrations indicated below.

B. Incubation Conditions

All incubations were carried out in Erlenmeyer flasks in a water bath at 37°, with shaking. Concentrations of components in the reaction mixture were as follows: 5 per cent Ehrlich ascites carcinoma cells, 15 mM glucose, and 33.25 mM sodium bicarbonate. Total incubation volumes were 5 mls when radioactive purine base was substrate, and 2 or 50 mls, depending on the experiment, when ³H-labeled 6MeMPR was substrate.

Cells, buffer, glucose, and 6MeMPR were incubated for 15 to 20 minutes in an atmosphere of 95 per cent oxygen - 5 per cent carbon dioxide in flasks 5 times the total incubation volume, and the reaction was initiated by addition of radioactive substrate.

C. Conditions with Guanine-¹⁴C as Precursor

Cells were collected as described and suspended in 1 volume of Fisher's medium containing sodium bicarbonate and glucose. Concentrations of components in the reaction mixtures were the same as described previously except for sodium bicarbonate, which was 22.91 mM, and Fisher's medium, which was 61.8 per cent of the total incubation volume. Guanine-¹⁴C, 6MeMPR, and Fisher's medium containing buffer and glucose were incubated for 20 minutes as described previously, and the reaction was initiated by the addition of 0.5 ml of the cell suspension.

D. Termination of Incubations and Extraction of Cells

1. Radioactive Purine Base as Substrate

At appropriate times after addition of radioactive precursor, 0.5 ml samples of the incubation mixture were pipetted into chilled 12 x 75 mm plastic tubes containing 20 μ l of cold 42% perchloric acid. The contents of the tube were mixed well and chilled for 10 minutes at 4°. The acidic mixtures were adjusted to pH 7 to 8 by the addition of 10 μ l of 1 M Tris HCl buffer, pH 7.4 and 20 μ l of potassium hydroxide.

Samples were frozen to achieve maximum precipitation of potassium perchlorate and the latter was removed by centrifugation. A portion of the supernatant solution was used for isolation of radioactive bases, nucleosides, and nucleotides by thin-layer chromatography. Isolation of nucleotides was begun on the same day as the incubation.

2. ^3H -labeled 6MeMPR as Substrate

Two mls of the incubation mixture were removed and centrifuged in a chilled tube. The cells were washed once by suspension in 1 ml of cold Racker's buffered saline containing 25 μ g/ml of heparin and 1 mg/ml of glucose. They were then extracted with 0.5 ml of cold 2.6 M perchloric acid. After removal of denatured protein by centrifugation, the supernatant solution was neutralized with KOH. Potassium perchlorate was removed by centrifugation, the supernatant solution was adjusted to constant volume with distilled water, and radioactivity of a portion was measured.

III METHODS FOR STUDIES in vivo

A. Collection of Cells

Only mice weighing between 23 and 27 g were used for transplantation, and cells were collected on the fourth day of tumor growth. Collection of cells was the same as that described above except that the collecting solution contained no glucose and the cells were immediately centrifuged for 1 minute at 2,000 rpm without preceeding washes. These and subsequent manipulations were carried out at 4°.

For isolation of hypoxanthine in vivo, cells were collected and extracted in 10 mls of buffered saline containing 250 µg of heparin, and 26 mmoles of perchloric acid.

B. Extraction of Cells

After removal of ascitic fluid, cells were extracted twice with 0.4 M perchloric acid. Denatured protein was removed by centrifugation, and the combined supernatants were neutralized with KOH. After removal of potassium perchlorate by centrifugation, the supernatant solution was frozen and FGAR or ³H-labeled 6MeMPR 5'-phosphate was measured.

IV MEASUREMENT OF TUMOR CELL MASS

Cells from mice bearing 7 or 4 day ascites tumors were collected as described above. Cells were extracted with 0.4 M perchloric acid, and after the supernatant solution was removed by centrifugation, the

precipitate was washed with acetone and dried in vacuo. The dried tumor cells weighed 120.6 and 100.2 mg/ml wet packed cell volume for cells collected as described on the seventh and fourth day of tumor growth respectively. Each value is the average of 3 to 6 determinations.

V ANALYTICAL PROCEDURES

A. Isolation of Purine Ribonucleotides by One-Dimensional Thin-Layer Chromatography

The following is a modification of the method of Crabtree (33). A piece of Whatman 3 MM paper, 28 x 20.5 cm was folded to be approximately 2 to 2.5 cm long and 20.5 cm wide and then attached to the top 0.5 to 0.75 cm of a PEI-cellulose on Mylar sheet with staples so that the leading edge of the wick was in contact with the solvent. After the yellow impurities were removed from the sheet onto the wick by washing the sheet in 4 M formate buffer for 6 hours, the plates were dried and salt was removed by development in methanol-water (1:1) for 8 to 14 hours, and the plates dried again. Plates were washed and developed in an ascending direction in glass tanks, 29 x 10 x 23 cm (LxWxH) containing 100 ml of solvent.

Three to four μ l of a single carrier solution containing 10 μ mole/ml each of NAD, IMP, XMP, AMP, ADP, ATP, GMP, GDP and GTP in water were placed on six 1-cm segments 2 cm apart on a line 1 cm from the bottom of the sheet. When the radioactive base precursor was adenine, hypoxanthine, or guanine, carrier adenine, hypoxanthine and inosine, or guanine and xanthine respectively were added to the

carrier solution as a visual check for contamination of the nucleotide fraction. Ten μ l of the neutralized perchloric acid extract were streaked on each 1-cm segment with drying between applications by a cold air stream. Both carrier and sample solution were kept at 4° during streaking.

After the radioactive base precursor was washed onto the wick by washing the plate in methanol-water (1:1) for 10 to 12 hours, (or in two 4 to 6-hour periods separated by drying) the plate was dried and then developed by quickly transferring it to increasing concentrations of formic acid/sodium formate buffers, pH 3.4 without drying between as follows: 0.5 M formate to a line 2.5 cm from the origin, then 2.0 M formate to a line 7 cm above the origin, and finally to the top of the plate with 4.0 M formate until the solvent front had gone onto the wick about 0.5 cm.

The plate was dried and after the nucleotide areas were outlined with pencil under U.V. light, they were cut out, and radioactivity was measured.

B. Isolation of Purine Bases and Ribonucleosides by Two-Dimensional Thin-Layer Chromatography

Three to four μ l of each carrier solution, containing 10 μ moles per ml of each compound shown were placed, in the order given below, in a 0.5 to 1 cm diameter spot centered 2 cm from the bottom and left edge of the sheet.

Carrier
Solution

I	guanine, xanthine, uric acid in 0.1 N NaOH
II	guanosine, xanthosine in 0.03 N NaOH
III	hypoxanthine, inosine, adenosine in water
IV	NAD, IMP, XMP, AMP, ADP, ATP, GMP, GDP, GTP in water

(Solutions I to III were stored at 4° and made fresh every two weeks. Solution IV, with or without adenine hypoxanthine and inosine is stable for months when stored frozen).

After 10 µl of neutralized perchloric acid extract were spotted, spots were dried with a stream of warm air and the first dimension was developed in glass tanks containing 100 mls of acetonitrile-ammonium acetate, 0.1 M, pH 7 - ammonia (60:30:10). Each plate was air dried and 1 cm of cellulose was scraped off at the bottom and top of the plate (with reference to the first dimension). Following rotation of the plate so that the origin spot was in the lower right corner, the plate was developed twice with 1-butanol-methanol-water-ammonia (60:20:20:1 to the top of the plate with air drying in between runs. The plate was dried, the ultra-violet absorbing areas were cut out, and radioactivity was measured.

C. Isolation of FGAR

FGAR was isolated by the method of Henderson (21). The neutralized perchloric acid extract was poured onto 10 x 30 to 40 mm columns of Dowex-1-formate, X8, defined 200-400 mesh in glass tubing, the bottom of which contained glass wool and the top of which was connected to 50 ml Erlenmeyers. After the sample had drained completely

into the resin column, glycine and phosphoribosyl-glycineamide were eluted with 0.5 M formic acid. FGAR was eluted with 15 ml of 4 M formic acid, and the chilled eluates were evaporated to dryness in vacuo in 50 ml beakers over sodium hydroxide flakes. Radioactivity in FGAR was measured as described.

D. Isolation of Nucleic Acid Adenine and Guanine

The perchloric acid insoluble fraction of cells was washed twice with 0.4 M perchloric acid, and the nucleic acids were hydrolyzed by heating in a boiling water bath for 60 minutes. After cooling, the precipitate was removed by centrifugation and each supernatant solution was poured onto a 5 x 10 mm column of Dowex-50W-H⁺, X10, de-fined 200 - 400 mesh in glass tubing, the bottom of which contained glass wool, and the top of which was connected to a 50 ml Ehrlenmeyer. The column was washed with 5 ml of 1 N HCl then with 2.5 ml of water, and eluted with 5 ml of 6 N HCl. The eluate was evaporated to dryness in vacuo. The dried residue was dissolved in two 50 to 100 μ l portions of 0.1 N HCl and the entire sample was spotted in a 3 cm streak on Whatman No. 1 paper. The chromatogram was developed descending in isopropanol-HCl for 20 hours. (Isopropanol:HCl:water (65:16.4:18.6 by volume)). Adenine and guanine were located with ultra-violet light, cut out, and radioactivity was measured. Toluene was removed from the paper by acetone elution (34). After drying, the paper was eluted with 4 mls of 0.1 N HCl in a tube for 16 hours. Absorbancy of the eluate was read at 263 nm for adenine, and 275 nm for guanine in a spectrophotometer and specific activities were calculated.

E. Conversion of Hypoxanthine Derivatives to Hypoxanthine and its Isolation

The acid-soluble fraction from perchloric acid extraction was placed in a tube in a boiling water bath for 1 hour to convert nucleotides and nucleosides to purine bases. The acid-insoluble material from hot perchloric acid extraction was removed by centrifugation, and the supernatant solution was evaporated to dryness in vacuo. The dried residue was dissolved in a known volume of 0.1 N HCl and a portion was spotted on Whatman No. 1 paper with carriers adenine and hypoxanthine. These were separated by development in Na_2HPO_4 for 6 hours. Hypoxanthine was further purified by elution with 0.1 N HCl followed by chromatography as above. Radioactivity of hypoxanthine was measured as described.

VI RADIOACTIVITY MEASUREMENTS

A. Scintillation Fluids

All radioactivity measurements were made in a liquid scintillation counter using either toluene, Bray's, or polyether 611 scintillation liquid phosphor. The composition of toluene phosphor is: PPO, 4 g; POPOP, 0.1 g; toluene, 1 liter. Bray's scintillation liquid contains: naphthalene, 60 g; PPO, 4 g; POPOP, 0.2 g; absolute methanol, 100 ml; ethylene glycol, 20 ml; and p-dioxane to give a total volume of 1 liter. The composition of polyether 611 scintillation liquid is: p-dioxane, 750 ml; anisole, 125 ml; dimethoxyethane 125 ml; PPO, 15 g; POPOP, 625 mg.

B. Methods for Measuring Radioactivity

1. Measurement of Purine Bases, Ribonucleosides and Ribonucleotides

The ultra-violet absorbing area of the Mylar Chromatography sheet was measured for radioactivity with the thin-layer facing the top of a counting vial containing 18 ml of toluene phosphor solution. Counting efficiency is approximately 72 per cent. Although quenching (as measured by the channels ratio method) is higher for PEI-cellulose than for cellulose layers, the channels ratio for all samples counted on the same layer is similar, so that the radioactivity of individual samples on the same layer can be compared directly (33).

2. Measurement of FGAR

FGAR was dissolved in 1 ml of water and the radioactivity of 0.5 ml was measured in 4.5 ml of Bray's scintillation liquid.

3. Measurement of 6MeMPR 5'-phosphate

A 0.5 or 0.1 ml aliquot (depending on the total radioactivity) of the neutralized perchloric acid extract was measured for radioactivity in 9 ml of polyether 611 scintillation liquid. The counting efficiency of ^3H -labeled 6MeMPR was 18.2 and 22.2 per cent when 0.5 and 0.1 ml volumes respectively of neutralized perchloric acid extract were present. Quenching by the 0.5 ml aliquot was 19.3 per cent.

4. Measurement of Adenine, Guanine, and Hypoxanthine
Isolated from Experiments in vivo

Adenine, guanine, and hypoxanthine areas of the chromatogram were measured for radioactivity in a counting vial containing 18 ml of toluene phosphor solution.

EFFECT OF 6MeMPR ON THE METABOLISM OF PURINE BASES

I. CHOICE OF 6MeMPR CONCENTRATIONS FOR STUDIES in vitro

A. Introduction

In order to study the biochemical mechanism of tumor growth inhibition of a drug in vitro, one should use drug concentrations related to those which cause growth inhibition in vivo. Before studying the effect of 6MeMPR on the metabolism of purine bases, therefore, it was necessary to know the concentration of 6MeMPR 5'-phosphate found in tumor cells in vivo at growth-inhibitory drug doses.

When 6MeMPR was injected i.p. daily for four days into tumor-bearing mice, the dose of 6MeMPR which caused a 50 per cent reduction in the quantity of tumor cells was found to be 0.75 to 1.0 mg/Kg (Dr. T. Sawa, personal communication). This ID₅₀ value was obtained when the first dose of 6MeMPR was given 24 hours after the i.p. implantation of $1 \text{ to } 2 \times 10^6$ cells into mice weighing 23 to 27 g, and the cells were collected and tumor volumes recorded two hours after the last dose of 6MeMPR. Under these conditions the concentrations of 6MeMPR 5'-phosphate 2 and 3.5 hours after the last drug injection were 137 and 131 nmoles per ml packed cells, respectively. This latter value was later confirmed in our experiments, in which the concentration of 6MeMPR 5'-phosphate 3.5 hours after the last dose of 6MeMPR was 121 nmoles per ml packed cells.

B. Results and Discussion

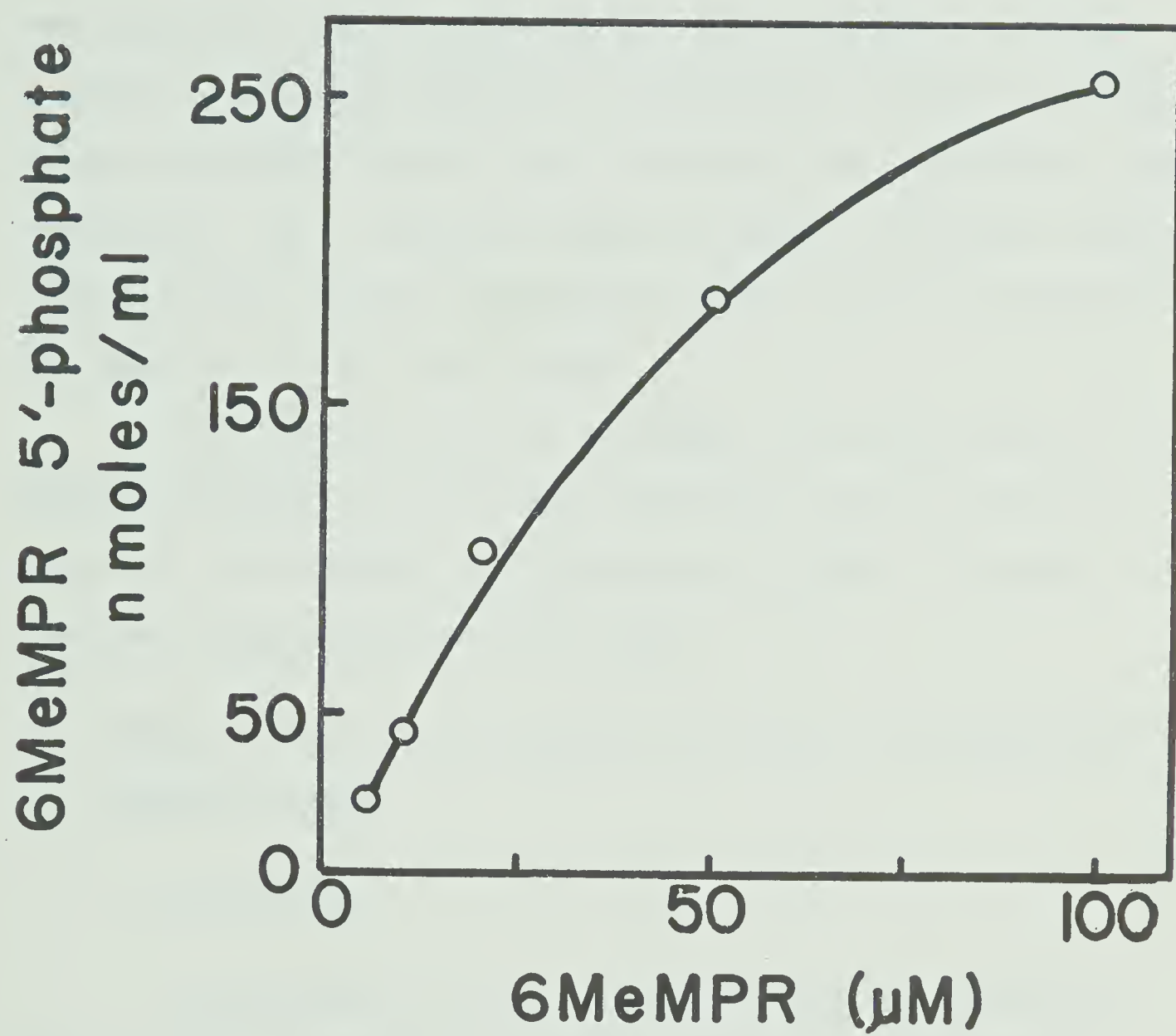
1. Effect of 6MeMPR Concentration on 6MeMPR 5'-phosphate Formation by Ehrlich Ascites Tumor Cells in vitro

Chart 5 shows 6MeMPR 5'-phosphate concentrations in Ehrlich ascites tumor cells incubated in vitro with various initial extracellular concentrations of 6MeMPR. One hundred and thirty-one nmoles of 6MeMPR 5'-phosphate per ml packed cells, the concentration of 6MeMPR 5'-phosphate found in vivo 3.5 hours after an ID₅₀ dose of 6MeMPR (0.75 mg/Kg i.p. for four days) was found at an initial extracellular 6MeMPR concentration of 32 μ M.

The ID₅₀ value of 6MeMPR was not however, firmly established before this project was begun, and 6MeMPR concentrations of 20 and 100 μ M were chosen for the study of the effect of 6MeMPR on the metabolism of purine bases on the basis of Sawa's preliminary experiments. Some experiments were also done using 2 μ M 6MeMPR, but this concentration gave values of 6MeMPR 5'-phosphate much lower than those found in vivo at an ID₅₀ dose of 6MeMPR. The results just described suggest that drug concentrations of 20 and 100 μ M 6MeMPR are reasonable for studies in vitro.

Chart 5. Effect of 6MeMPR concentration on 6MeMPR 5'-phosphate formation by Ehrlich ascites tumor cells in vitro.

Ehrlich ascites tumor cells, 5% by volume, were incubated in a 10 ml Ehrlenmeyer flask at 37° with shaking in an atmosphere of 95% oxygen - 5% carbon dioxide in 2 ml of Fisher's medium containing 15 mM glucose and 33.25 mM sodium bicarbonate. After 20 min, various amounts of ³H-labeled 6MeMPR (10 µc/µMole) were added, and the incubation continued for 30 min. Each flask represents the mean of separate analyses of duplicate flasks in one experiment.



2. Effect of Incubation Time on 6MeMPR 5'-phosphate Formation by Ehrlich Ascites Tumor Cells

The concentrations of 6MeMPR 5'-phosphate shown in Chart 5 were determined after an incubation period of 30 minutes, and it was necessary to determine whether the concentration of 6MeMPR 5'-phosphate changed appreciably over the 3 hour incubation period planned for other experiments. Chart 6 shows the amounts of 6MeMPR 5'-phosphate formed between 0.5 and 3.0 hours when Ehrlich ascites cells were incubated in the presence of 20 and 100 μM 6MeMPR.

At a concentration of 20 μM 6MeMPR, the concentration of 6MeMPR 5'-phosphate did not change appreciably between 0.5 and 3.0 hours. At 100 μM 6MeMPR, the concentration of 6MeMPR 5'-phosphate increased slightly between 1 and 3 hours.

II. EFFECT OF 6MeMPR ON THE METABOLISM OF ADENINE, HYPOXANTHINE AND GUANINE in vitro

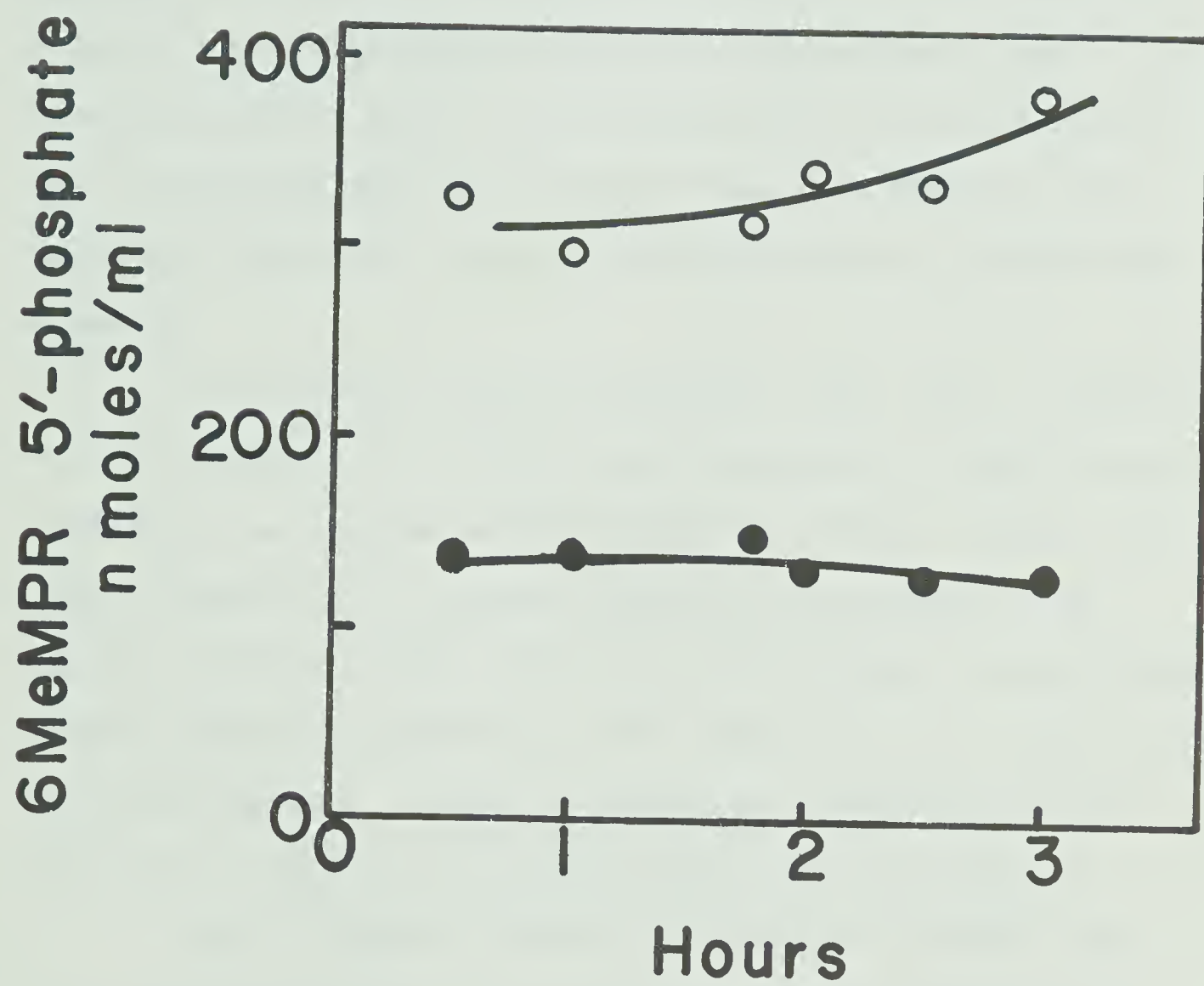
A. Introduction

Identification of those biochemical reactions depicted in Chart 4 which are inhibited by 6MeMPR was based on drug-induced increases and decreases in the amounts of metabolites formed from radioactive purine bases. In the following experiments only major changes in amounts of radioactive metabolites which occur consistently within a given time course and in more than one experiment are considered significant.

Chart 6. Effect of incubation time on 6MeMPR 5'-phosphate formation by Ehrlich ascites tumor cells.

Tumor cells were incubated as described in Chart 5, except that a 250-ml flask was used, and the total volume was 50 ml. At various times, 2 ml portions were removed. Each point represents the mean of separate analyses of duplicate samples in one experiment.

● — ● 6MeMPR:20 μ M
○ — ○ 6MeMPR:100 μ M.



B. Results

The results of the experiments on the effect of 6MeMPR on the metabolism of adenine- ^{14}C , hypoxanthine- ^{14}C and guanine- ^{14}C are presented in Tables 1 to 5. Tables 1, 3, and 5 show the effect of 20 μM 6MeMPR on the metabolism of radioactive purine bases during a 3 hour incubation period. Charts 7 to 9 are based on the data from Tables 1 and 3. Some experiments were also done using 2 μM 6MeMPR, but no appreciable drug effects were found and these data are therefore not presented.

In an attempt to detect drug effects which might be undetected or only marginal at 20 μM 6MeMPR, the concentration of 6MeMPR for some experiments, was extended to 100 μM as shown in Tables 2 and 4. In Table 4, because total nucleotide synthesis was decreased in the presence of 100 μM 6MeMPR, values for metabolites whose formation depends on total nucleotide synthesis are also expressed as a per cent of total nucleotide synthesis. Changes in the values of metabolites due to drug action in Table 4 are therefore discussed on this basis.

Table 6 summarizes the major drug effects of 6MeMPR. The following sections of the text discuss the effect of 6MeMPR on metabolites shown in Tables 1 to 5.

1. Effect of 6MeMPR on XMP, Xanthosine and Xanthine
Formed from Adenine and Hypoxanthine

The largest and most consistent effect of 6MeMPR in all experiments was a decrease in the amount of xanthosine formed from adenine- ^{14}C and hypoxanthine- ^{14}C . Chart 7 shows that xanthosine formation from adenine- ^{14}C and hypoxanthine- ^{14}C at 2.5 hours was decreased by 74 and 65 per cent respectively in the presence of 20 μM 6MeMPR. In the presence of 100 μM 6MeMPR, xanthosine formation from adenine- ^{14}C and hypoxanthine- ^{14}C at 2 hours was decreased by 66 and 91 per cent, respectively (Tables 2 and 4).

The quantity of XMP formed was not altered by 20 μM 6MeMPR, but that formed from hypoxanthine- ^{14}C was inhibited by 100 μM 6MeMPR (Table 4).

There was no change in the amount of xanthine formed from either adenine- ^{14}C or hypoxanthine- ^{14}C in the presence of 6MeMPR.

2. Effect of 6MeMPR on Guanine Nucleotides, Guanosine
and Guanine Formed from Adenine and Hypoxanthine

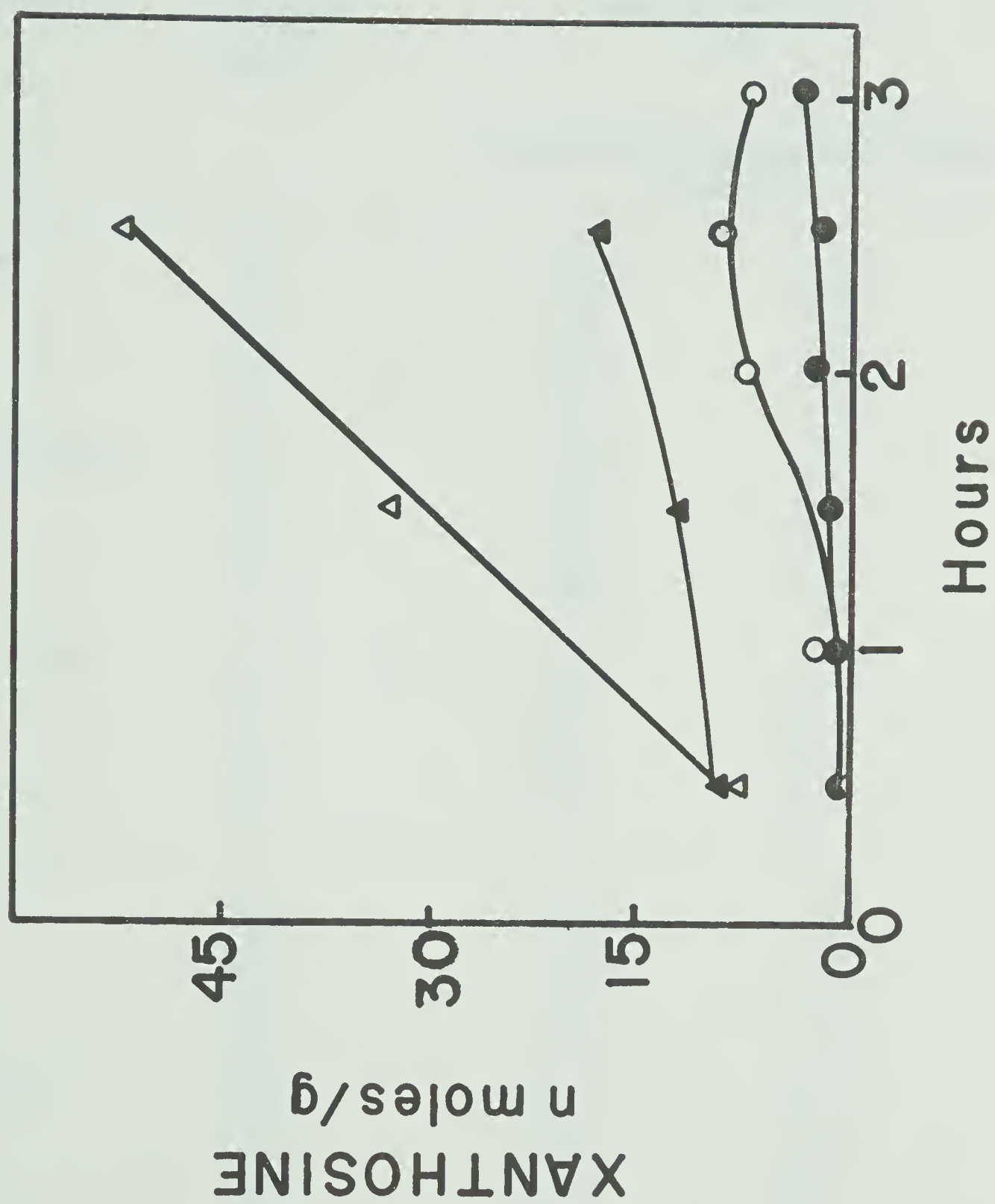
At 20 μM 6MeMPR, total guanine nucleotide synthesis from hypoxanthine- ^{14}C was decreased 16 to 36 per cent between 1.5 and 3 hours (Table 3). When adenine- ^{14}C was used as precursor, the effect of 6MeMPR on total guanine nucleotide synthesis was inconsistent (Table 1). In another experiment not recorded here, total guanine nucleotides formed from adenine- ^{14}C in the presence of 20 μM 6MeMPR

Chart 7. Effect of 6MeMPR on xanthosine formed from adenine- ^{14}C and hypoxanthine- ^{14}C .

These data are plotted from the results presented in Tables 1 and 3.

○——○ Adenine- ^{14}C :control
●——● Adenine- ^{14}C :plus 20 μM 6MeMPR

△——△ Hypoxanthine- ^{14}C :control
▲——▲ Hypoxanthine- ^{14}C :plus 20 μM 6MeMPR.



Effect of 20 μ M 6MeMPR on the Metabolism of Adenine- ^{14}C

METABOLITE	INCUBATION TIME (hours)	6MeMPR CONCENTRATION (μ M)	
		0	20
		RADIOACTIVE METABOLITE CONCENTRATION (nmoles/g)	
XMP	0.5	0.514	0.771
	1.0	0.807	0.954
	1.5	1.32	1.28
	2.0	2.24	1.32
	2.5	1.10	1.65
	3.0	2.86	1.25
Xanthosine	0.5	0.479	0.702
	1.0	2.11	1.24
	1.5	1.28	1.50
	2.0	7.34	2.84
	2.5	9.00	2.30
	3.0	7.56	3.57
Xanthine	0.5	1.21	1.75
	1.0	4.72	5.90
	1.5	10.1	10.1
	2.0	13.7	13.1
	2.5	16.9	17.5
	3.0	19.1	19.5
GMP	0.5	3.60	2.75
	1.0	3.16	2.02
	1.5	2.42	2.64
	2.0	1.76	1.50
	2.5	3.41	3.41
	3.0	5.65	3.86
GDP	0.5	10.8	18.4
	1.0	10.6	9.80
	1.5	25.1	13.8
	2.0	14.1	30.2
	2.5	16.3	61.2
	3.0	38.3	17.5
GTP	0.5	16.7	8.84
	1.0	33.9	22.9
	1.5	46.1	39.4
	2.0	42.4	40.8
	2.5	39.7	38.6
	3.0	50.8	37.1

Effect of 20 μM 6MeMPR on the Metabolism of Adenine- ^{14}C

METABOLITE	INCUBATION TIME (hours)	6MeMPR CONCENTRATION (μM)	
		0	20
		RADIOACTIVE METABOLITE CONCENTRATION (nmoles/g)	
Guanosine	0.5	1.08	1.18
	1.0	1.75	1.91
	1.5	2.84	2.71
	2.0	1.50	1.50
	2.5	1.40	1.53
	3.0	1.15	1.50
Guanine	0.5	0.925	0.670
	1.0	1.21	1.63
	1.5	1.85	1.34
	2.0	1.60	1.28
	2.5	0.606	1.18
	3.0	1.12	3.73
IMP	0.5	2.50	2.86
	1.0	15.0	16.9
	1.5	23.9	24.5
	2.0	28.9	33.9
	2.5	35.2	40.1
	3.0	44.2	44.7
Inosine	0.5	6.83	9.09
	1.0	10.6	15.7
	1.5	10.8	18.4
	2.0	6.86	15.9
	2.5	5.26	11.9
	3.0	6.35	9.47
Hypoxanthine	0.5	27.1	35.1
	1.0	58.0	81.9
	1.5	47.7	71.2
	2.0	37.1	68.6
	2.5	31.9	68.7
	3.0	27.7	53.2

Effect of 20 μM 6MeMPR on the Metabolism of Adenine- ^{14}C

METABOLITE	INCUBATION TIME (hours)	6MeMPR CONCENTRATION (μ M)	
		0	20
		RADIOACTIVE METABOLITE CONCENTRATION (nmoles/g)	
AMP	0.5	128	210
	1.0	34.9	36.9
	1.5	15.1	19.5
	2.0	23.2	12.1
	2.5	23.9	28.6
	3.0	10.6	41.1
ADP	0.5	321	361
	1.0	217	228
	1.5	154	180
	2.0	152	140
	2.5	160	153
	3.0	104	184
ATP	0.5	755	582
	1.0	887	954
	1.5	952	1000
	2.0	871	829
	2.5	729	763
	3.0	760	702
Adenosine	0.5	2.33	3.83
	1.0	1.63	2.55
	1.5	2.39	2.17
	2.0	2.62	3.64
	2.5	2.78	3.35
	3.0	4.27	6.99
Adenine	0.5	266	326
	1.0	13.5	28.8
	1.5	3.99	3.67
	2.0	3.32	3.22
	2.5	2.55	3.00
	3.0	14.1	3.54
Uric Acid	0.5	3.19	4.50
	1.0	11.1	14.9
	1.5	20.7	18.4
	2.0	25.1	32.8
	2.5	33.9	34.7
	3.0	30.0	33.8

TABLE 1 (Cont'd.)

Effect of 20 μ M 6MeMPR on the Metabolism of Adenine- ^{14}C

Tumor cells were incubated as described in Chart 5 with 20 μ M 6MeMPR, except a 25 ml Ehrlenmeyer flask was used, and the total volume was 5 ml. After 20 min adenine- ^{14}C was added to a final concentration of 100 μ M. Portions were removed for analysis at various times. Each value represents a single determination.

TABLE 2

Effect of 100 μM 6MeMPR on the Metabolism of Adenine- ^{14}C

METABOLITE	6MeMPR CONCENTRATION (μM)	
	0	100
	RADIOACTIVE METABOLITE CONCENTRATION (nmoles/g)	
XMP	1.74	1.20
Xanthosine	8.47	2.89
GMP	4.21	3.28
GDP	19.6	15.0
GTP	33.9	24.7
IMP	4.05	4.90
Inosine	33.0	46.2
Hypoxanthine	129	210
AMP	36.0	33.8
ADP	213	193
ATP	871	852
Adenosine	3.96	2.82
Adenine	103	142
Uric Acid	14.2	14.4

Tumor cells were incubated as described in Table 1 with 100 μM 6MeMPR. After 20 min, adenine- ^{14}C was added to a final concentration of 100 μM . Portions were removed for analysis 2 hr later. Each value represents the average of 3 determinations in one experiment.

Effect of 20 μM 6MeMPR on the Metabolism of Hypoxanthine- ^{14}C

METABOLITE	INCUBATION TIME (hours)	6MeMPR CONCENTRATION (μM)	
		0	20
		RADIOACTIVE METABOLITE CONCENTRATION (nmoles/g)	
XMP	0.5	3.90	4.20
	1.0	3.50	3.94
	1.5	7.49	5.28
	2.0	7.14	9.57
	2.5	21.0	15.0
	3.0	7.80	28.6
Xanthosine	0.5	7.95	9.25
	1.5	33.2	12.5
	2.5	52.6	18.3
Xanthine	0.5	23.0	36.0
	1.5	84.0	84.7
	2.5	111	95.4
GMP	0.5	4.59	2.90
	1.0	6.24	12.3
	1.5	6.71	6.32
	2.0	8.01	8.57
	2.5	7.79	9.79
	3.0	8.62	9.09
GDP	0.5	11.4	12.3
	1.0	20.7	33.5
	1.5	23.2	26.8
	2.0	28.4	21.1
	2.5	23.6	30.7
	3.0	27.0	31.1
GTP	0.5	56.2	52.7
	1.0	108	115
	1.5	138	192
	2.0	128	75.0
	2.5	127	95.5
	3.0	117	66.6

Effect of 20 μ M 6MeMPR on the Metabolism of Hypoxanthine- 14 C

METABOLITE	INCUBATION TIME (hours)	6MeMPR CONCENTRATION (μ M)	
		0	20
		RADIOACTIVE METABOLITE CONCENTRATION (nmoles/g)	
Guanosine	0.5	13.8	16.6
	1.5	7.25	6.65
	2.5	5.58	5.31
Guanine	0.5	2.17	3.54
	1.5	1.44	2.64
	2.5	2.94	2.17
IMP	0.5	66.1	67.3
	1.0	93.4	190
	1.5	221	193
	2.0	252	251
	2.5	270	267
	3.0	308	253
Inosine	0.5	56.3	75.4
	1.5	28.0	36.0
	2.5	13.3	15.5
Hypoxanthine	0.5	1250	1230
	1.5	242	214
	2.5	67.9	62.6
AMP	0.5	9.66	8.66
	1.0	*	45.3
	1.5	12.8	23.0
	2.0	34.3	35.6
	2.5	41.3	30.4
	3.0	43.9	33.9
ADP	0.5	36.8	40.6
	1.0	86.2	141
	1.5	73.9	92.7
	2.0	118	112
	2.5	88.4	129
	3.0	143	97.1

Effect of 20 μM 6MeMPR on the Metabolism of Hypoxanthine- ^{14}C

METABOLITE	INCUBATION TIME (hours)	6MeMPR CONCENTRATION (μM)	
		0	20
		RADIOACTIVE METABOLITE CONCENTRATION (nmoles/g)	
ATP	0.5	218	286
	1.0	471	533
	1.5	598	522
	2.0	591	419
	2.5	311	600
	3.0	587	372
Adenosine	0.5	12.6	10.8
	1.5	12.8	7.55
	2.5	9.52	8.58
Adenine	0.5	0.367	0.501
	1.5	2.87	1.50
	2.5	2.10	1.77
Uric Acid	0.5	56.1	45.2
	1.5	188	160
	2.5	236	184

Tumor cells were incubated as described in Table 1 with 20 μM 6MeMPR. After 20 min, hypoxanthine- ^{14}C was added to a final concentration of 100 μM . Portions were removed for analysis at various times. Each point represents a single determination.

* unreliable value.

Effect of 100 μ M 6MeMPR on the Metabolism of Hypoxanthine- 14 C

METABOLITE	6MeMPR CONCENTRATION			
	(0 100 μ M)		(0 100 μ M)	
	RADIOACTIVE METABOLITE CONCENTRATION (nmoles/g)		CONCENTRATION (% of total nucleotides)	
XMP	6.58	2.83	0.859	0.497
Xanthosine	137	8.87	17.9	1.56
GMP	9.70	6.18	1.27	1.09
GDP	26.3	18.1	3.43	3.18
GTP	80.4	58.4	10.5	10.3
IMP	10.8	11.1	1.41	1.95
Inosine	70.0	147		
Hypoxanthine	258	799		
AMP	47.1	42.7	6.15	7.50
ADP	102	84.2	13.3	14.8
ATP	483	345	63.1	60.6
Adenosine	15.1	12.0	1.97	2.11
Adenine	3.2	1.94	0.418	0.341
Uric Acid	32.2	22.8		

Tumor cells were incubated as described in Table 1 with 100 μ M 6MeMPR. After 20 min, hypoxanthine- 14 C was added to a final concentration of 100 μ M. Portions were removed for analysis 2 hr later. Each value represents the average of 3 determinations in one experiment.

were decreased 21 per cent, as they were at 100 μM 6MeMPR in Table 2. At 100 μM 6MeMPR there was no significant decrease in total guanine nucleotides formed from hypoxanthine- ^{14}C , but interpretation of results was complicated by the fact that total nucleotide synthesis was decreased (Table 4).

There appeared to be no change in the amount of guanosine and guanine formed from either adenine- ^{14}C or hypoxanthine- ^{14}C in the presence of 6MeMPR.

3. Effect of 6MeMPR on IMP, Inosine, and Hypoxanthine Formed from Adenine and Hypoxanthine

There appeared to be no change in the amount of IMP formed from adenine- ^{14}C or hypoxanthine- ^{14}C in the presence of 6MeMPR.

Chart 8 shows that in the presence of 20 μM 6MeMPR there was a significant increase in inosine formed from adenine- ^{14}C between 0.5 and 3.0 hours. Between 1 and 2.5 hours there was 33 to 56 per cent more inosine formed in the presence of 6MeMPR as compared to the control samples. At 100 μM 6MeMPR, inosine formed from adenine- ^{14}C was increased by 29 per cent (Table 2). In Table 3, at 20 μM 6MeMPR, inosine formed from hypoxanthine- ^{14}C was increased slightly at all times (25 and 22 per cent at 0.5 and 1.5 hours). At 100 μM 6MeMPR, inosine formed from hypoxanthine- ^{14}C was increased by 52 per cent (Table 4).

As can be seen in Chart 9, in the presence of 20 μM 6MeMPR, hypoxanthine formed from adenine- ^{14}C was significantly increased (29 to 54 per cent) between 1 and 3 hours. At 100 μM 6MeMPR, hypoxanthine formed from adenine- ^{14}C was increased 39 per cent (Table 2).

Chart 8. Effect of 6MeMPR on inosine formed from adenine-¹⁴C.

These data are plotted from the results presented in Table 1.

o—o Adenine-¹⁴C:control
e—* Adenine-¹⁴C:plus 20 μ M 6MeMPR.

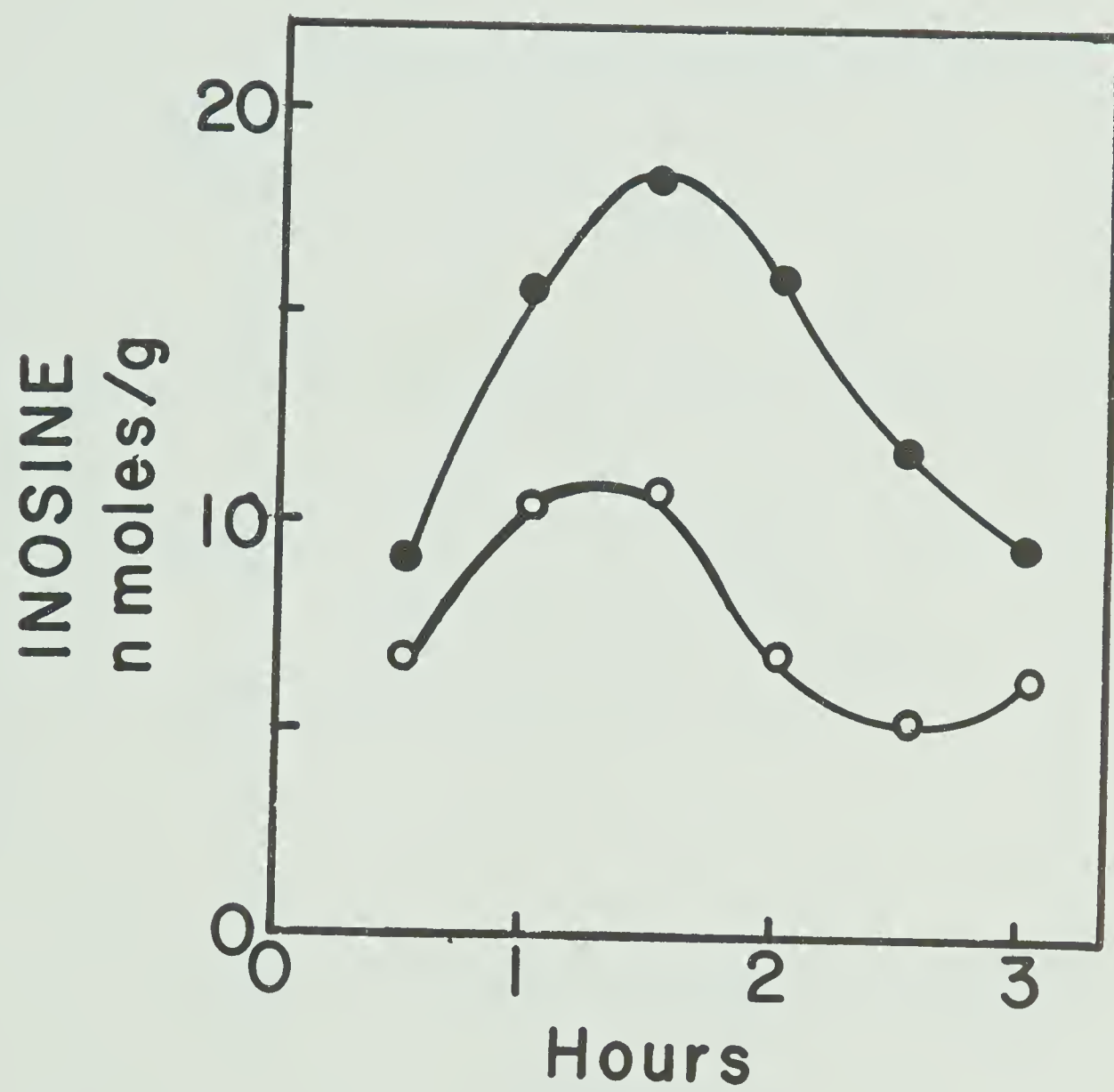
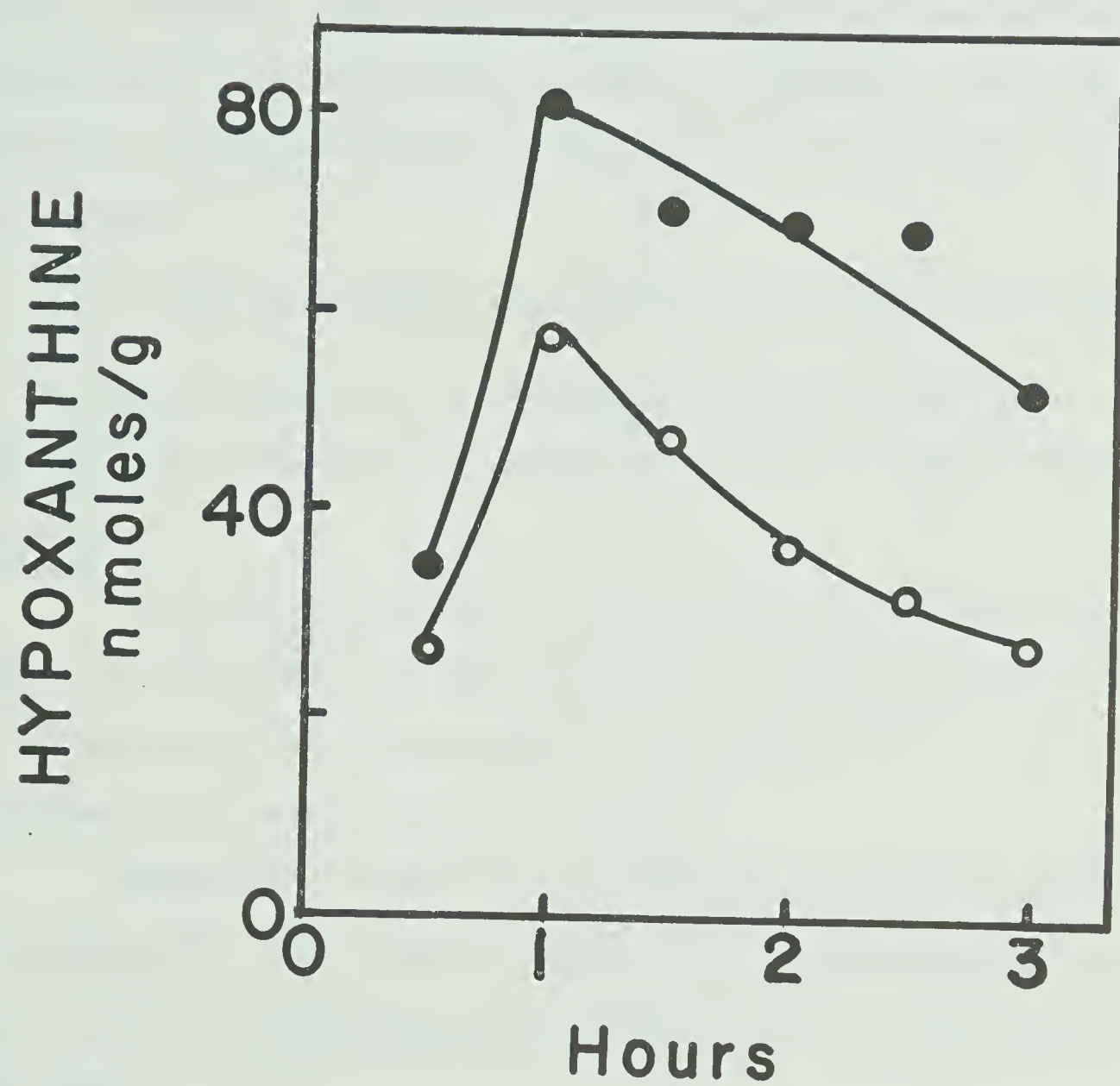


Chart 9. Effect of 6MeMPR on hypoxanthine formed from adenine-¹⁴C.

These data are plotted from the results presented in Table 1.

○——○ Adenine-¹⁴C:control
●——● Adenine-¹⁴C:plus 20 μM 6MeMPR.



4. Effect of 6MeMPR on Adenine Nucleotides, Adenosine, Adenine and Uric Acid Formed from Adenine and Hypoxanthine

There appeared to be no significant changes in the amounts of adenine nucleotides, adenosine and adenine formed from adenine- ^{14}C and hypoxanthine- ^{14}C in the presence of 6MeMPR. In addition, there were no significant changes in amounts of uric acid formed from either of these two precursors.

5. Effect of 6MeMPR on Purine Metabolites Formed from Guanine

In the presence of 20 μM 6MeMPR, xanthosine formed from guanine- ^{14}C was increased 25 to 42 per cent between 1.5 and 2.5 hours (Table 5).

ATP formed from guanine- ^{14}C was decreased 50 to 74 per cent between 0.5 and 3 hours, and ADP formed from guanine- ^{14}C was decreased 45 to 53 per cent at all times except 1 and 2 hours, where it had approximately the same value as the control.

6MeMPR had no effect on any of the other metabolites formed from guanine- ^{14}C . The effects of 6MeMPR on the metabolism of guanine- ^{14}C are at present not completely understood, and were not further explored in this study.

C. Discussion

1. IMP Dehydrogenase as a Site of Inhibition by 6MeMPR

The data presented above indicate that IMP dehydrogenase is one biochemical site of inhibition of 6MeMPR. Although no changes were found in the amounts of IMP and XMP, 6MeMPR caused a significant

Effect of 20 μ M 6MeMPR on the Metabolism of Guanine- 14 C

METABOLITE	INCUBATION TIME (hours)	6MeMPR CONCENTRATION (μ M)	
		0	20
		RADIOACTIVE METABOLITE CONCENTRATION (nmoles/g)	
XMP	0.5	55.4	58.9
	1.0	111	102
	1.5	118	98
	2.0	115	109
	2.5	143	119
	3.0	119	142
Xanthosine	0.5	13.2	10.9
	1.0	12.2	14.6
	1.5	14.8	19.8
	2.0	24.0	32.9
	2.5	22.2	38.1
Xanthine	0.5	460	420
	1.0	653	627
	1.5	593	722
	2.0	840	78.7
	2.5	767	671
GMP	0.5	31.7	27.5
	1.0	42.3	54.2
	1.5	33.3	40.8
	2.0	50.1	39.4
	2.5	29.3	40.0
	3.0	49.2	22.3
GDP	0.5	82.2	92.0
	1.0	93.9	134
	1.5	66.4	28.5
	2.0	58.2	70.2
	2.5	66.6	47.9
	3.0	54.5	43.7
GTP	0.5	265	317
	1.0	322	303
	1.5	328	171
	2.0	367	335
	2.5	312	279
	3.0	260	185

TABLE 5 (cont'd.)

Effect of 20 μM 6MeMPR on the Metabolism of Guanine- ^{14}C

METABOLITE	INCUBATION TIME (hours)	6MeMPR CONCENTRATION (μM)	
		0	20
		RADIOACTIVE METABOLITE CONCENTRATION (nmoles)	
Guanosine	0.5	50.7	51.3
	1.0	19.6	14.9
	1.5	56.3	6.50
	2.0	12.7	13.4
	2.5	18.8	6.25
Guanine	0.5	551	518
	1.0	184	150
	1.5	18.6	15.3
	2.0	4.33	5.63
	2.5	3.47	6.87
IMP	0.5	33.9	25.2
	1.0	13.7	14.7
	1.5	44.8	43.5
	2.0	44.0	46.0
	2.5	33.0	49.3
	3.0	53.9	26.0
Inosine	0.5	5.32	4.09
	1.0	4.39	7.43
	1.5	5.32	6.81
	2.0	7.30	7.06
	2.5	6.99	6.56
Hypoxanthine	0.5	3.59	2.91
	1.0	3.10	2.48
	1.5	2.04	2.04
	2.0	3.28	4.09
	2.5	2.41	1.86
AMP	0.5	47.2	33.9
	1.0	46.4	48.4
	(1.5)	*	*
	2.0	17.8	14.8
	2.5	37.0	17.9
	3.0	11.3	41.5

TABLE 5 (cont'd.)

Effect of 20 μM 6MeMPR on the Metabolism of Guanine- ^{14}C

METABOLITE	INCUBATION TIME (hours)	6MeMPR CONCENTRATION (μM)	
		0	20
		RADIOACTIVE METABOLITE CONCENTRATION	
ADP	0.5	8.85	4.84
	1.0	9.52	8.04
	1.5	8.41	3.94
	2.0	9.45	9.37
	2.5	14.2	7.81
	3.0	13.4	6.77
ATP	0.5	15.25	6.47
	1.0	16.6	8.18
	1.5	15.0	3.87
	2.0	18.9	8.3
	2.5	19.4	7.66
	3.0	17.1	5.65
Adenosine	0.5	29.0	54.8
	1.0	46.9	11.9
	1.5	5.69	14.0
	2.0	11.9	5.6
	2.5	9.84	7.24
Adenine	0.5	0.681	0.867
	1.0	0.805	1.11
	1.5	2.41	1.80
	2.0	1.61	0.743
	2.5	0.990	1.05
Uric Acid	0.5	75.5	66.5
	1.0	135	121
	1.5	158	126
	2.0	157	153
	2.5	161	142

Tumor cells were incubated with 20 μM 6MeMPR and 100 μM guanine- ^{14}C . (See methods section for modifications of the conditions described in Table 1). Portions were removed for analysis at various times. Each point represents a single determination.

* Unreliable value.

TABLE 6

Major Changes in Amounts of Metabolites Formed from Adenine- ^{14}C and
Hypoxanthine- ^{14}C in the Presence of 20 and 100 μM 6MeMPR

METABOLITE	PRECURSOR	6MeMPR CONCENTRATION (μM)	CHANGE % OF CONTROL	TIME (hours)	REFERENCE TABLE	CHART
Xanthosine	Adenine- ^{14}C	20	-74.4	2.5	1	7
		100	-65.9	2.0	2	
	Hypoxanthine- ^{14}C	20	-65.2	2.5	3	7
		100	-91.3	2.0	4	
Total Guanine Nucleotides	Adenine- ^{14}C	100	-20.8	2.0	2	
	Hypoxanthine- ^{14}C	20	-15.6 to -36.0	1.5 to 3.0	3	

(Continued)

TABLE 6 (Cont'd.)

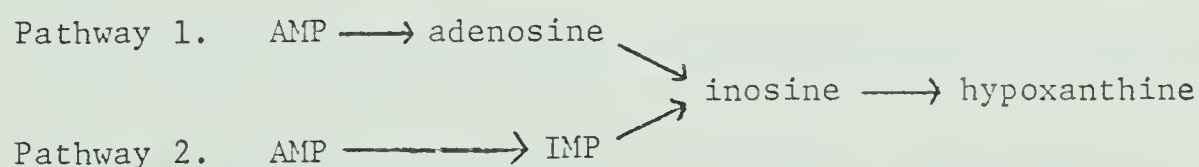
Major Changes in Amounts of Metabolites Formed from Adenine- ^{14}C and Hypoxanthine- ^{14}C in the Presence of 20 and 100 μM 6MeMPR

METABOLITE	PRECURSOR	6MeMPR CONCENTRATION (μM)	CHANGE % OF CONTROL	TIME (Hours)	REFERENCE TABLE	CHART
Inosine	Adenine- ^{14}C	20	+32.5 to +55.8	1.0 to 2.5	1	8
		100	+28.6	2.0	2	
	Hypoxanthine- ^{14}C	20	+25.3, +22.2	0.5, 1.5	3	
		100	+52.4	2.0	4	
Hypoxanthine	Adenine- ^{14}C	20	+29.2 to +53.6	1.0 to 3.0	1	9
		100	+38.7	2.0	2	

increase in the base and nucleoside formed upon dephosphorylation of IMP derived from adenine- ^{14}C , and a concurrent decrease in the nucleoside formed upon dephosphorylation of XMP derived either from adenine- ^{14}C or hypoxanthine- ^{14}C . It has been found previously (33) that IMP and XMP do not accumulate in these cells, but are readily dephosphorylated. Nucleoside monophosphates may be dephosphorylated to nucleosides by non-specific acid or alkaline phosphatases and by specific 5'-nucleotidases.

Crabtree found that when adenine- ^{14}C was used as precursor, the synthesis of other purine nucleosides and bases was quite low during early times of incubation, but increased later, indicating that these compounds arise from breakdown of nucleotides formed from adenine- ^{14}C rather than via metabolism of adenine per se. These results are in accord with the observations of other investigators that (a) Ehrlich cells do not contain adenine deaminase and (b) adenine can not be converted to adenosine by the purine nucleoside phosphorylase of Ehrlich cells.

AMP may be catabolized to hypoxanthine by two routes as follows:



It is believed that the major route of AMP degradation in Ehrlich ascites tumor cells in vitro is via Pathway 2 (33). When adenine- ^{14}C is used as precursor, AMP, and consequently IMP is formed.

As more IMP is formed, it may be either utilized for AMP and GMP synthesis or degraded to inosine and hypoxanthine.

When 6MeMPR inhibits IMP dehydrogenase, the IMP which is not converted to XMP is catabolized to inosine and hypoxanthine, so that no significant change in total adenine nucleotides is apparent. However, a decrease in xanthosine, the dephosphorylation product of XMP, can be seen.

2. Hypoxanthine-Guanine Phosphoribosyltransferase as a Site of Inhibition by 6MeMPR

The observed increase in hypoxanthine formed from adenine- ^{14}C and decrease in xanthosine formed from adenine- ^{14}C and hypoxanthine- ^{14}C in the presence of 6MeMPR might at first be thought to be due to inhibition of hypoxanthine-guanine phosphoribosyltransferase. However, the observation that total nucleotide synthesis from hypoxanthine- ^{14}C was not inhibited at 20 μM but only at 100 μM 6MeMPR, and that 20 μM 6MeMPR did not inhibit total nucleotide synthesis from guanine- ^{14}C , indicate that hypoxanthine-guanine phosphoribosyltransferase is not a sensitive site of inhibition by moderate concentrations of 6MeMPR.

3. Other Effects of 6MeMPR on the Metabolism of Purine Bases

As well as the major effects of 6MeMPR on the metabolism of purine bases, there were some other drug effects which were either small or were not consistent from one experiment to another. These remain to be further studied.

D. Conclusion

The most significant effect of 6MeMPR on the metabolism of purine bases in vitro observed in these studies is inhibition of IMP dehydrogenase. Whether this biochemical effect of 6MeMPR is related to the mechanism by which it inhibits tumor growth requires demonstration in vivo.

III. EFFECT of 6MeMPR on IMP DEHYDROGENASE in vivo

A. Introduction

6MeMPR appears to inhibit IMP dehydrogenase in Ehrlich ascites tumor cells in vitro. To test the possible relationship of this drug action to its growth inhibitory properties, it is first necessary to establish that 6MeMPR inhibits this reaction in sensitive tumor cells in vivo at chemotherapeutic doses. Two approaches to this problem were made; in neither case was clear-cut evidence obtained for inhibition of IMP dehydrogenase in vivo, but because of the technical and conceptual difficulties encountered, further studies are warranted.

B. Results and Discussion

1. Effect of 6MeMPR on Incorporation of Hypoxanthine-¹⁴C into Nucleic Acid Guanine

(i) Introduction

The conversion of hypoxanthine-¹⁴C to nucleic acid guanine requires IMP dehydrogenase, and significant inhibition of this enzyme by 6MeMPR would be expected to cause a decrease in the total

radioactivity and specific activity of the product. Unfortunately, the interpretation of results is complicated by the inhibition of purine biosynthesis de novo which is known to occur under the conditions of the experiment, and by the possibility that other enzymes, including hypoxanthine-guanine phosphoribosyltransferase may also be inhibited by 6MeMPR.

Inhibition of purine biosynthesis de novo may have two effects on hypoxanthine metabolism. The accumulation of PP-ribose-P which is known to occur may increase nucleotide synthesis from hypoxanthine. In addition, decreases in adenine and guanine nucleotide pool sizes due to inhibition of purine biosynthesis de novo will result in less than normal dilution of radioactivity. As attempts to control these factors, the incorporation of hypoxanthine- ^{14}C into nucleic acid adenine was also measured, as was that of adenine- ^{14}C into nucleic acid adenine, and of guanine- ^{14}C into nucleic acid guanine.

(ii) Results and Discussion

When hypoxanthine- ^{14}C , adenine- ^{14}C , or guanine- ^{14}C were incorporated into nucleic acid adenine and guanine in the presence of 6MeMPR, the specific radioactivity of the product was increased (Table 7). The increase in specific activity is presumably due to reduction of nucleotide pool sizes due to inhibition of purine biosynthesis de novo by 6MeMPR. In addition, there was an increase in total radioactivity which is due in part to an increase in availability of PP-ribose-P which stimulates the phosphoribosyltransferase reactions.

Effect of 6MeMPR on Incorporation of Hypoxanthine- ^{14}C
into Nucleic Acid Guanine

PRECURSOR	6MeMPR DOSE (mg/Kg)	PRODUCT	
		Adenine cpm/ μMole	Guanine cpm/ μMole
Hypoxanthine- ^{14}C	0	49,639	79,408
	6.8	86,510	130,987
Adenine- ^{14}C	0	105,677	14,831
	6.8	298,772	46,627
Guanine- ^{14}C	0	458	31,281
	6.8	5,155	39,471

Mice bearing 4-day Ehrlich ascites tumors were injected i.p. with 6.8 mg/Kg of 6MeMPR. Two hr later they were injected with one of the following radioactive purine bases: hypoxanthine- ^{14}C (0.18 μmole); adenine- ^{14}C (0.20 μmole); guanine- ^{14}C (0.03 μmole). Three hours after injection of 6MeMPR cells were collected, and nucleic acid adenine and guanine were isolated.

The absolute increase in specific activity of nucleic acid guanine following drug treatment was no less when hypoxanthine- ^{14}C was precursor (+50,000) than when guanine- ^{14}C was precursor (+8,000). Likewise, the relative change in specific activity of nucleic acid guanine following drug treatment was no less when hypoxanthine- ^{14}C was precursor (Treated/Control = 1.65) than when guanine- ^{14}C was precursor (Treated/Control = 1.26). Although 6MeMPR may be inhibiting IMP dehydrogenase in vivo, due to the complicating factors mentioned previously, such an effect is not apparent from these data.

2. Effect of 6MeMPR on the Conversion of Adenine- ^{14}C into Hypoxanthine and its derivatives in vivo

(i) Introduction

Increased synthesis of hypoxanthine and inosine from adenine- ^{14}C was noted in vitro as an effect of inhibition of IMP dehydrogenase by 6MeMPR. An attempt was therefore made to do a similar experiment in vivo.

(ii) Results and Discussion

Mice bearing 4-day Ehrlich ascites tumors were injected i.p. with 3.8 mg/Kg of 6MeMPR. Ten microcuries of adenine- ^{14}C (50 mCi/mMole) were injected 2.5 hours later, and the cells were removed and extracted with perchloric acid 3 hours after injection of 6MeMPR. Purine nucleosides and nucleotides were converted to bases

by hot acid hydrolysis, and total radioactivity was determined in extracts from drug treated and control mice.

6MeMPR had no effect on total hypoxanthine, inosine and IMP formed from adenine- ^{14}C in vivo. However, if 6MeMPR is also inhibiting purine biosynthesis de novo, the increased PP-ribose-P levels may increase nucleotide synthesis from adenine- ^{14}C and increase reutilization of the IMP catabolite, hypoxanthine. The amount of hypoxanthine which accumulates because of inhibition of XMP dehydrogenase in vivo may therefore not be apparent.

C. Conclusion

While 6MeMPR was shown to inhibit IMP dehydrogenase in vitro, such an effect of 6MeMPR on this enzyme was not demonstrated in vivo.

CHAPTER IV

EFFECT OF 6MeMPR ON PURINE BIOSYNTHESIS DE NOVO

A. Introduction

As mentioned in Chapter I, 6MeMPR has been shown to be a powerful feedback inhibitor of purine biosynthesis de novo in Ehrlich ascites cells in vitro. Whether the growth inhibitory effect of 6MeMPR is a consequence of this feedback inhibition remains to be proven by showing that 6MeMPR inhibits purine biosynthesis de novo in vivo at dosage schedules which inhibit tumor growth.

The Ehrlich ascites cells of mice were treated with azaserine to inhibit the enzyme phosphoribosyl-formylglycineamidine synthetase (EC 6.3.5.3) in the pathway of purine biosynthesis de novo. The amount of FGAR formed from glycine-¹⁴C under these conditions is a measure of the rate of the pathway. Decreases in the amount of FGAR in the presence of exogenously supplied 6MeMPR have been shown to be due to inhibition of this pathway.

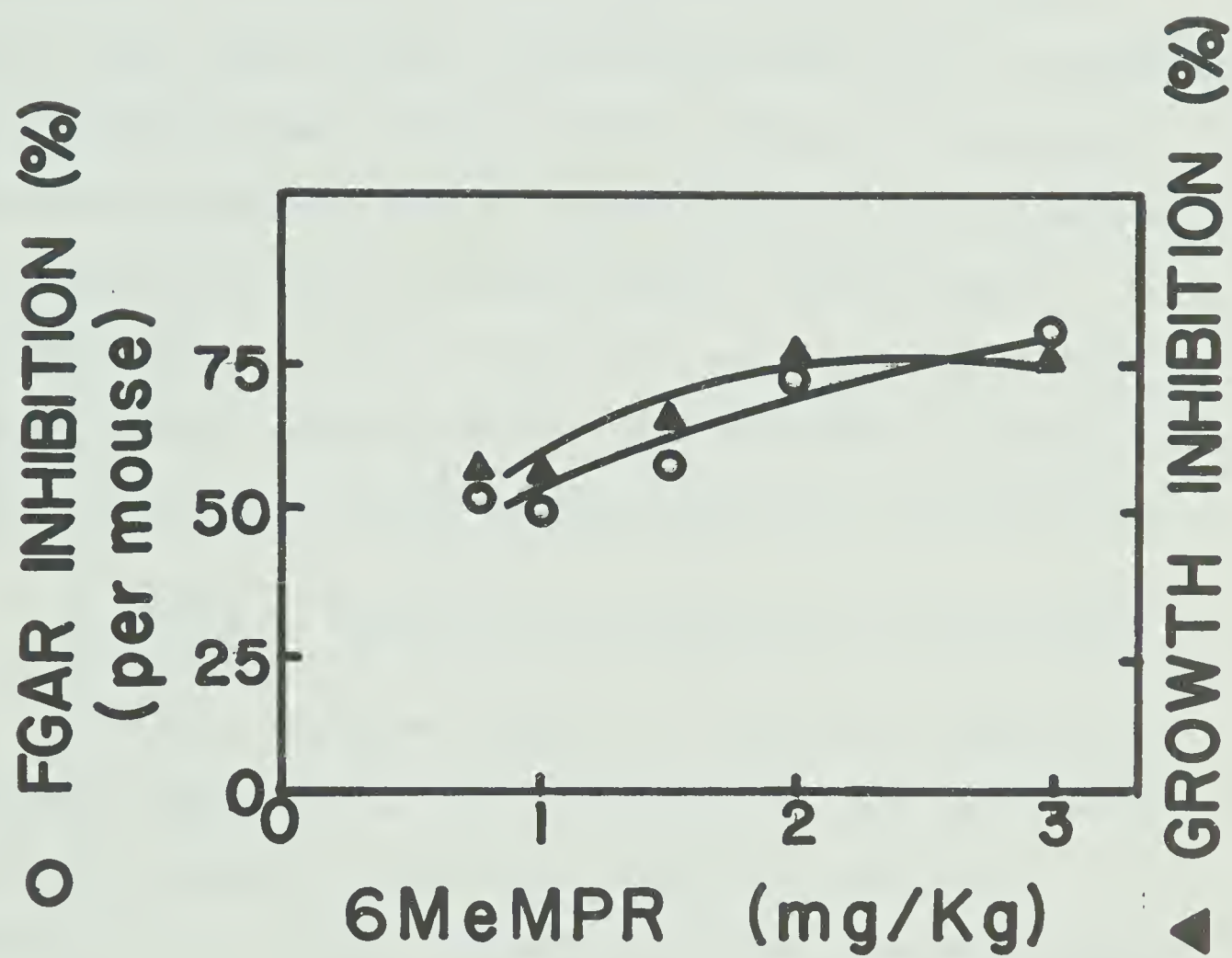
B. Results and Discussion

1. Effect of 6MeMPR on FGAR Accumulation in Treated Mice

When mice were treated on four successive days with 6MeMPR, 0.75 to 3 mg/Kg, as described in Chart 10, the per cent inhibition of tumor growth was 58 to 76 per cent. The ID₅₀ for 6MeMPR was 0.75 to 1 mg/Kg, which agrees with that reported by Sawa (T. Sawa, personal communication).

Chart 10. Effect of 6MeMPR on FGAR accumulation in Ehrlich ascites tumor cells of treated mice.

Mice implanted i.p. with 1 to 2×10^6 Ehrlich ascites tumor cells were treated with various doses of 6MeMPR daily for 4 days beginning 24 hours after implantation. Two hr after the last drug treatment, $10 \mu\text{g}$ of azaserine was injected i.p., and 30 min later, $2 \mu\text{Moles}$ of glycine- ^{14}C ($2 \mu\text{c}$). One hr after injection of glycine- ^{14}C , tumor cells were removed, packed cell volumes were determined and the FGAR contents of perchloric acid extracts were measured. Each point represents an average of 3 to 6 determinations in one experiment.



The per cent inhibition of FGAR accumulation for the same dosage schedule of 6MeMPR was 53 to 82 per cent, based on total FGAR per mouse, which suggests that the decrease in FGAR may be simply a consequence of a decrease in volume of tumor cells. However the interpretation of these data is complicated by the fact that the volume of tumor cells is less in drug-treated animals than in controls. Thus the relative amount of glycine which is taken up by the tumor cells and the dilution of this by endogenous non-radioactive glycine may both vary from control to treated animals. Either or both phenomena, neither of which was controlled, may make a simple interpretation of these results uncertain. Thus studies were extended to determine the effect of 6MeMPR on FGAR accumulation in untreated mice.

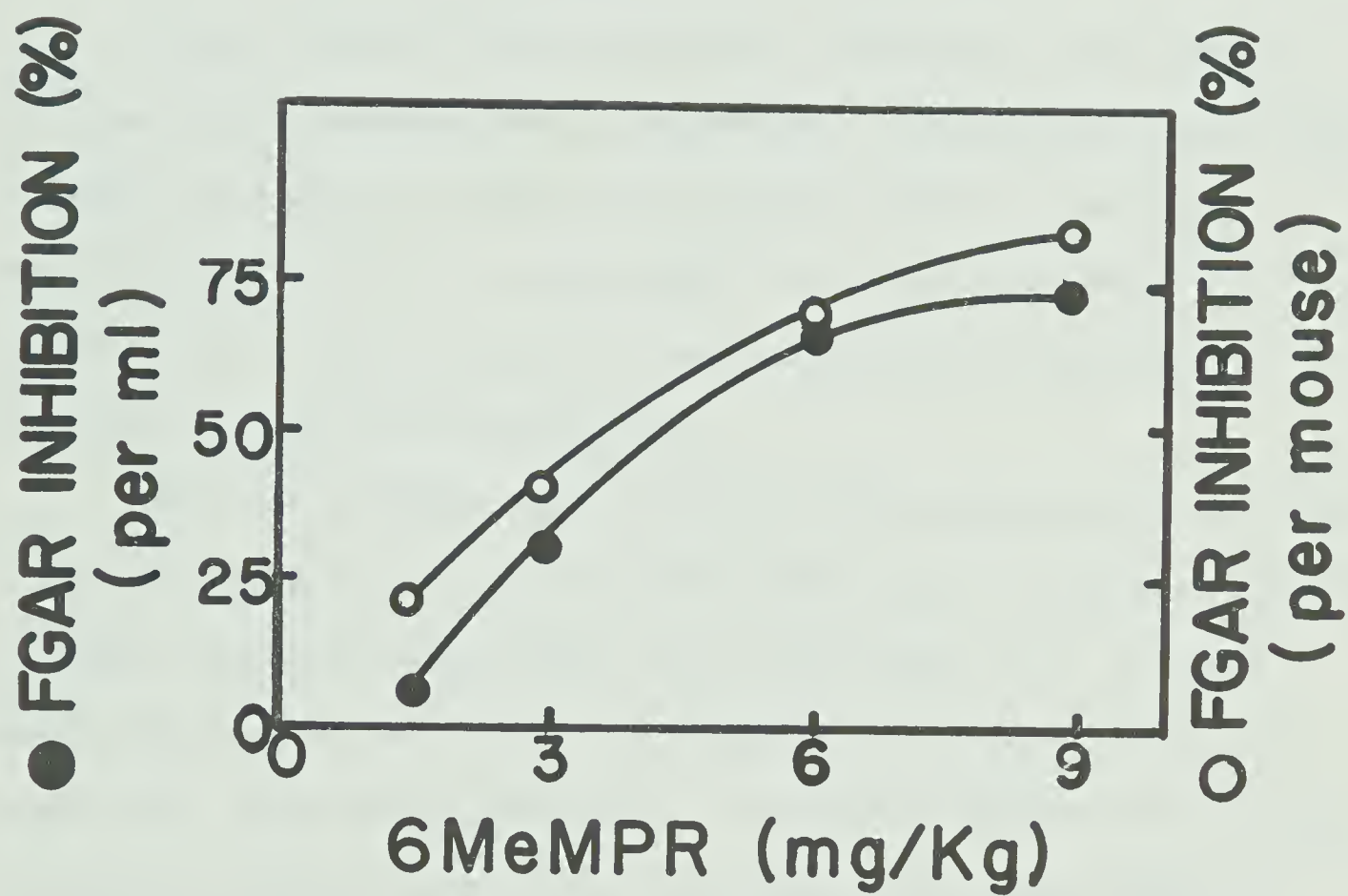
2. Effect of 6MeMPR on FGAR Accumulation in Untreated Mice

When mice were given a single i.p. injection of 6MeMPR on the fourth day of tumor growth, (i.e. "untreated mice") and the packed cell volumes were measured 3.5 hours later (Chart 11), there was no inhibition of tumor growth. Similarly, in another experiment using 10 mice per dose, no inhibition of tumor growth was detected 7 days after tumor implantation, when mice received a single dose of 6MeMPR, 0.75 to 12 mg/Kg on the fourth day of tumor growth.

Chart 11 shows that when untreated mice received one i.p. injection of 6MeMPR, 1.5 to 9 mg/Kg, FGAR accumulation was inhibited 21 to 85 per cent, based on FGAR accumulation per mouse, and 6 to 73 per cent, based on FGAR accumulation per ml packed cells. Under the

Chart 11. Effect of 6MeMPR on FGAR accumulation in Ehrlich
ascites tumor cells of untreated mice.

Mice bearing 4 day ascites tumor cells were treated with a single
i.p. injection of various amounts of 6MeMPR. Mice were then
injected with azaserine and glycine-¹⁴C, and FGAR was determined
as described in Chart 10. Each point represents an average of
3 to 5 determinations in one experiment.



same conditions in a separate experiment, when untreated mice received one i.p. injection of 12 mg/Kg 6MeMPR, FGAR accumulation was inhibited 89 and 83 per cent based on FGAR accumulation per mouse and per ml packed cells respectively.

3. Comparison of 6MeMPR 5'-phosphate Formed in the Ehrlich Ascites Cells of Treated and Untreated Mice

Charts 12 and 13 show that 6MeMPR 5'-phosphate concentrations increased with increasing doses of 6MeMPR when expressed per mouse or per ml packed cells both in treated and untreated animals. When mice were treated with 0.75 to 3 mg/Kg 6MeMPR, there was more 6MeMPR 5'-phosphate per ml packed cells than when calculated per mouse due to inhibition of tumor growth (Chart 12).

The dose of 6MeMPR which inhibited FGAR accumulation 50 per cent in untreated mice was 3.8 and 4.4 mg/Kg 6MeMPR based on FGAR accumulation per mouse and per ml packed cells respectively (Chart 11). At these doses there were 100 and 118 nmoles of 6MeMPR 5'-phosphate per ml of packed cells respectively (Chart 13). At a dose of 6MeMPR which inhibited the growth of Ehrlich ascites cells 50 per cent (0.75 mg/Kg 6MeMPR in treated animals) (Chart 10), there were 121 nmoles of 6MeMPR 5'-phosphate per ml packed cells (Chart 12). These results agree with the values of 6MeMPR 5'-phosphate per ml packed cells reported by Sawa (124 and 131 nmoles per ml packed cells at doses of 3.8 and 0.75 mg/Kg 6MeMPR in untreated and treated animals respectively) (T. Sawa, personal communication).

Chart 12. Formation of 6MeMPR 5'-phosphate in Ehrlich ascites tumor cells of mice treated 4 times with ^3H -labeled 6MeMPR.

Mice implanted i.p. with 1 to 2×10^6 Ehrlich ascites tumor cells were treated with various doses of ^3H -labeled 6MeMPR ($8.2 \mu\text{c}/\mu\text{Mole}$) daily for 4 days beginning 24 hr after implantation. 3.5 hr after the last drug injection, tumor cells were removed and ^3H -labeled 6MeMPR 5'-phosphate was measured. Each point represents an average of 3 to 6 determinations in one experiment.

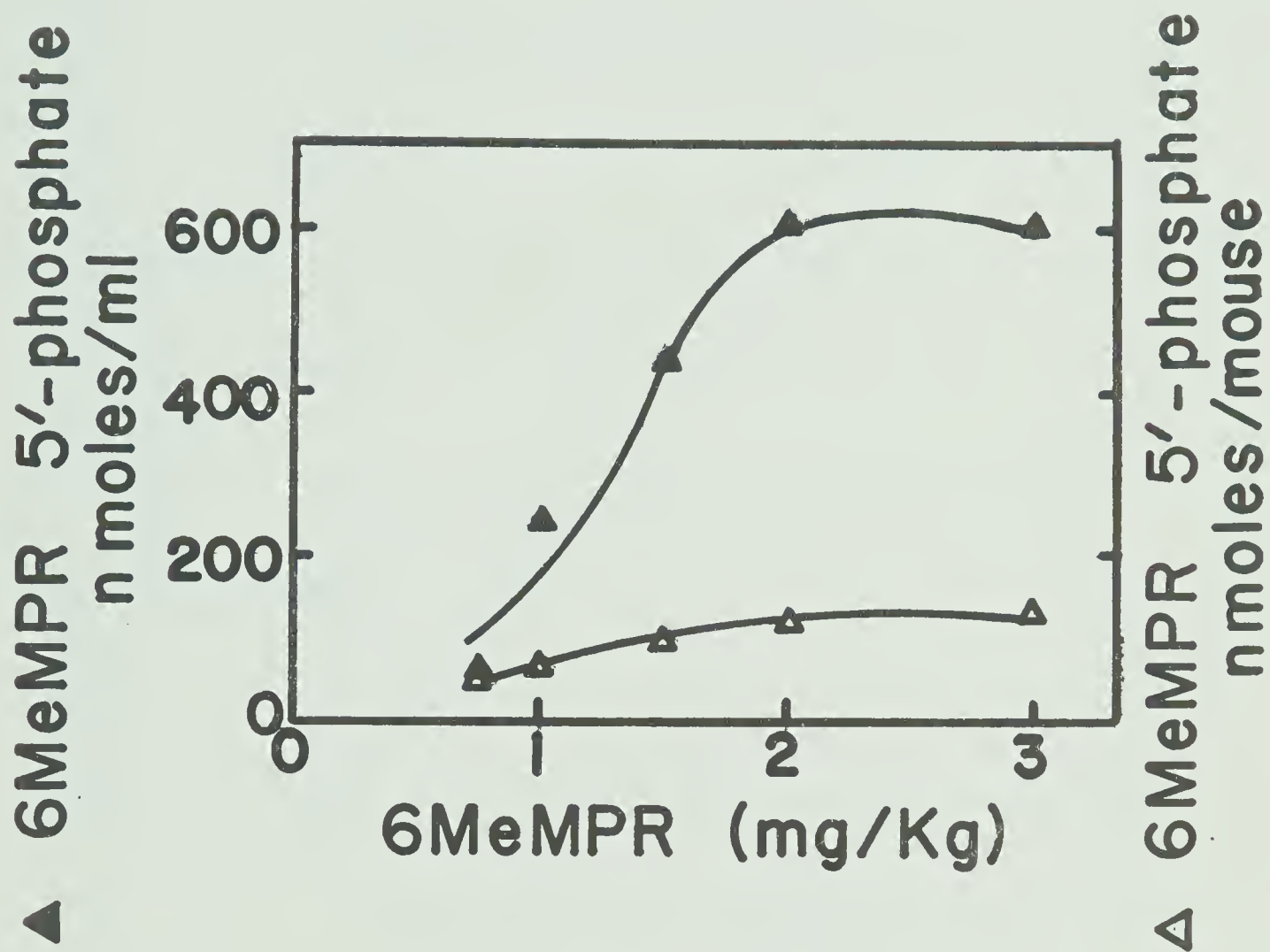
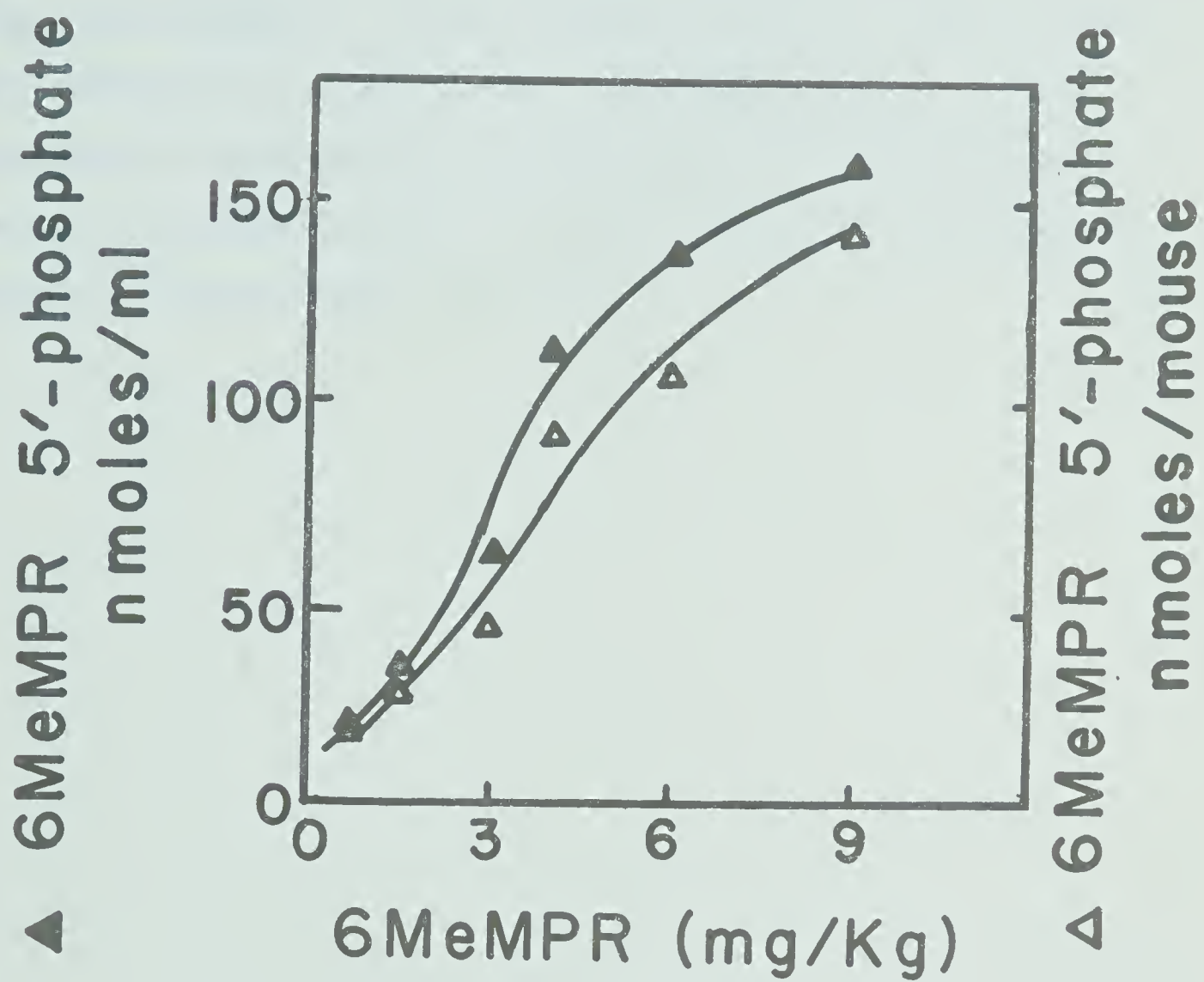


Chart 13. Formation of 6MeMPR 5'-phosphate in Ehrlich ascites tumor cells of mice treated once with ^3H -labeled 6MeMPR.

Mice bearing 4 day ascites tumor cells were treated with a single i.p. injection of various amounts of ^3H -labeled 6MeMPR ($8.2 \mu\text{c}/\mu\text{Mole}$). 3.5 hr after the last drug injection, tumor cells were removed and ^3H -labeled 6MeMPR 5'-phosphate was measured. Each point represents an average of 5 to 6 determinations in one experiment (except for the points at 3 and 4 mg/Kg, which represent data from 3 and 1 determinations respectively).



C. Conclusion

Approximately the same amount of 6MeMPR 5'-phosphate was found in the Ehrlich ascites tumor cells of mice who had received either an ID₅₀ dose of 6MeMPR, or a dose of 6MeMPR sufficient to cause a 50 per cent reduction in the rate of purine biosynthesis de novo. These findings are consistent with the hypothesis that the biochemical mechanism of action of 6MeMPR is inhibition of purine biosynthesis de novo in Ehrlich ascites tumor cells.

General Discussions and Conclusions

I. INTRODUCTION

One known biochemical effect of 6MeMPR is inhibition of purine biosynthesis de novo. A study of the effects of 6MeMPR on the metabolism of purine bases in vitro showed that it also inhibited IMP dehydrogenase, a biochemical effect of this drug not previously reported. Inhibition of IMP dehydrogenase was not however demonstrated in studies in vivo. The pharmacological significance of inhibition of purine biosynthesis de novo was deduced because of the correlation between the amount of 6MeMPR 5'-phosphate present in Ehrlich ascites tumor cells in vivo, the magnitude of inhibition of purine biosynthesis de novo, and the extent of tumor growth inhibition.

II. EFFECT OF 6MeMPR ON THE METABOLISM OF PURINE BASES

It has been found previously that IMP and XMP do not accumulate in Ehrlich ascites tumor cells, but are readily dephosphorylated (33). The conclusion that IMP dehydrogenase is inhibited by 6MeMPR in Ehrlich ascites tumor cells in vitro is based on the increase in inosine and hypoxanthine formed from adenine-¹⁴C, and the decrease in xanthosine formed from adenine-¹⁴C and hypoxanthine-¹⁴C.

At this time this research was started, there were no published studies of the effect of 6MeMPR on the metabolism of purine bases. Since then, Hill (28) published a study of the effects of 50 μ M 6MeMPR on purine metabolism in Adenocarcinoma 755 cells growing in suspension culture. Also he found that 6MeMPR did not inhibit partially purified hypoxanthine-guanine phosphoribosyltransferase from Adenocarcinoma 755 cells. In our studies there was no evidence for inhibition of hypoxanthine-guanine phosphoribosyltransferase with 20 μ M 6MeMPR, but at 100 μ M 6MeMPR total nucleotide synthesis from hypoxanthine-¹⁴C was decreased.

Hill found that 50 μ M 6MeMPR inhibited the conversion of radioactive adenine, hypoxanthine and guanine into total intracellular radioactivity. As a consequence, there was a reduction in total nucleoside di- and tri-phosphates, and of the incorporation of all three precursors into nucleic acids. No data on IMP and XMP levels were given, and it is not known whether these accumulate or are dephosphorylated in Adenocarcinoma 755 cells. Hill's base and nucleoside data are questionable because he discarded the incubation medium expected to contain bases and nucleosides. Cell constituents were analyzed in cells separated from the medium by centrifugation and washed with sodium chloride. Since these data are not comparable to data obtained in our studies from cells plus incubation medium, they will not be discussed further here.

III. EFFECT OF 6MeMPR ON PURINE BIOSYNTHESIS DE NOVO

Inhibition of purine biosynthesis de novo by 6MeMPR was correlated with the concentration of 6MeMPR 5'-phosphate present in the Ehrlich ascites tumor cells of mice, the magnitude of inhibition of purine biosynthesis de novo, and the extent of tumor growth inhibition. Azaserine (O-diazoacetyl-L-serine) is also known to be an inhibitor of purine biosynthesis de novo, and this inhibition has been related to its antitumor properties (35). These findings are consistent with the hypothesis that the biochemical mechanism of action of both azaserine and 6MeMPR is inhibition of purine biosynthesis de novo.

The mechanism of action of several purine antimetabolites has been defined by some workers as inhibition of purine biosynthesis de novo simply on the evidence that they do inhibit this pathway. Unless efforts are made to identify other biochemical effects, and studies are done to relate various biochemical effects to inhibition of tumor growth, such a conclusion is unwarranted.

Up to the present, the only report which has provided substantial evidence that the mechanism of action of 6MeMPR is inhibition of purine biosynthesis de novo was based on studies of the reversal of growth inhibition of Adenocarcinoma 755 cells (32). Reversal by adenine, hypoxanthine and aminoimidazole carboxamide is consistent with this hypothesis.

Another criterion which may assist in determining the biochemical mechanism of action of a drug is the duration of the biochemical inhibition. 6MeMPR 5'-phosphate was found to persist in Ehrlich ascites cells and in solid Ehrlich carcinoma as long as 24 hours after a single i.p. or subcutaneous injection of chemotherapeutic doses (Dr. T. Sawa, personal communication). If the duration of inhibition of purine biosynthesis de novo depends on the duration of 6MeMPR 5'-phosphate concentration, then this pathway may be conceivably inhibited longer than the time of measurement used in the present study (3.5 hours).

The fact that aminoimidazole carboxamide or hypoxanthine protected Adenocarcinoma 755 cells at low, but not at high concentrations of 6MeMPR indicates that this drug may have an additional site of inhibition at high doses (32). The identity and importance of this site of inhibition remain to be investigated.

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